

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 31/70, C07H 21/00, C12N 15/11		A1	(11) International Publication Number: WO 95/28942
			(43) International Publication Date: 2 November 1995 (02.11.95)
(21) International Application Number: PCT/US95/05179			(81) Designated States: AU, CA, JP, KR, NZ, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).
(22) International Filing Date: 25 April 1995 (25.04.95)			
(30) Priority Data:			
08/233,778 26 April 1994 (26.04.94) US			
08/238,177 4 May 1994 (04.05.94) US			
PCT/US94/13387 16 November 1994 (16.11.94) WO			Published With international search report.
(34) Countries for which the regional or international application was filed: US et al.			
08/350,431 5 December 1994 (05.12.94) US			
(71) Applicant: GENTA INCORPORATED [US/US]; 3550 General Atomics Court, San Diego, CA 92121 (US).			
(72) Inventors: GIACHETTI, Cristina; 946 Santa Estella, Solano Beach, CA 92075 (US). MARICH, James, E.; 2738 Dale Street, San Diego, CA 92104 (US). JAEGER, John, A.; Apartment 120, 5205 Genesee Cove, San Diego, CA 92122 (US).			
(74) Agents: WOLFF, Jessica, R. et al.; Lyon & Lyon, First Interstate World Center, Suite 4700, 633 West Fifth Street, Los Angeles, CA 90071-2066 (US).			
(54) Title: ANTISENSE OLIGOMERS FOR INHIBITING HUMAN PAPILLOMAVIRUSES			
(57) Abstract			
<p>Antisense oligomers complementary to and capable of hybridizing to a target region of an mRNA or pre-mRNA of a human papillomavirus are provided. Suitable target regions include sequences selected from a translation initiation codon, a splice donor site, a splice acceptor site, a coding region, a polyadenylation signal, a 3'-untranslated region or a 5'-untranslated region of an HPV gene.</p>			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

DESCRIPTIONAntisense Oligomers For Inhibiting
Human PapillomavirusesRelated Applications

This application is a continuation-in-part application of commonly-assigned United States Patent Application Serial No. 08/350,431, filed December 5, 1994; which is a continuation-in-part of commonly owned PCT application PCT/US94/13387 filed November 16, 1994; which is a continuation-in-part of commonly-assigned United States Patent Application Serial No. 08/238,177, filed May 4, 1994; which is a continuation-in-part of United States Patent Application Serial No. 08/233,778, filed April 26, 1994; which is a continuation-in-part of United States Patent Application Nos. 08/154,013 and 08/154,014, both filed November 16, 1993. The disclosures of all these applications are incorporated by reference.

Background of the Invention

Papillomaviruses are a group of small DNA viruses that induce warts (or papillomas) in a number of higher vertebrates, including humans. Although the viral nature of human warts has been known for many years, it has only recently been recognized that specific human papillomavirus ("HPV's") are closely linked with certain human cancers most notably human cervical carcinoma. This finding has focused interest on the specific subgroup of HPV's associated with genital infections.

The papillomaviruses are reported to be highly species specific and to induce squamous epithelial

tumors and fibroepithelial tumors in their natural hosts.

Bovine papillomavirus-1 (BPV-1) has been more fully characterized and has served as a prototype for studies
5 on the transformation and molecular biology of human papillomaviruses. Significant differences exist between BPV-1 and the HPV's.

Certain proteins have been proposed as targets for antisense therapy of HPV-caused conditions. Certain of
10 these targets have been proposed based on studies performed using BPV-1 and equivalent functions and properties have not necessarily been confirmed in all HPV's. In addition, other parameters to be considered in the selection of targets for antisense effect, such
15 as mRNA sequence homology of target proteins between BPV and a HPV or the mRNA's secondary structure (which strongly affect the accessibility of the target sequences) are low or are not known. This makes selection of HPV target sequences based on BPV target
20 sequences almost impossible.

HPV's associated with a risk for malignant progression include HPV-16, HPV-18, HPV-31 and HPV-33, among others. Genital HPV's not associated with a risk for malignant progression include HPV-6b and HPV-11.

25 In human papillomaviruses the E7 protein has been reported to have transcriptional modulating properties and to complex p105-RB, a product of the retinoblastoma tumor suppressor gene. In HPV-16 and HPV-18, E7 is reported to encode transforming proteins, proteins which
30 are multifunctional and possess both transcriptional modulatory and transformation properties similar to that of adenovirus E1A. It has been reported that the E7 proteins of all the genital associated HPV's can complex p105-RB in vitro regardless of their associated risk for
35 malignant progression. It has been proposed that the ability of the E7 proteins to complex p105-RB must

relate to a property common both to the high risk viruses (such as HPV-16 and HPV-18) and the low risk virus (such as HPV-6b and HPV-11), such as the induction of cellular proliferation, a feature of all benign
5 warts. In addition, since not all E7 proteins of the genital papillomaviruses can transform in tissue culture, it may be that the ability to complex p105-RB is not sufficient for transformation. Moreover, the mechanism by which E7 modulates transcription has not
10 been identified. (Fields, Virology, Vol. 2, p. 1639 (1990)).

The E6 gene and its protein is also reported as associated with transformation in high risk human papilloma viruses, through interactions with p53.
15 (Band, et al., EMBO Journal, Vol. 12, No. 5, pp. 1847-1852 (1993)).

Although certain HPV's such as HPV-6 and HPV-11 have been categorized as "low risk", in that patients infected with these HPV's appear to be at significantly
20 lower risk for malignant progression, it should be noted that such infections are not without risk for malignant progression and occasional tumors do contain these viral genomes that are transcriptionally active. Therefore, all genital HPV's should be considered capable of
25 causing serious conditions. Accordingly, detection and control of HPV infections, especially those caused by genital HPV's is important.

Summary of the Invention

The present invention is directed to antisense
30 oligomers which are complementary to and which are capable of hybridizing with a target sequence of a mRNA or pre-mRNA of an human papillomavirus. In particular, provided are antisense oligomers which are complementary to and which hybridize with a portion of a mRNA or pre-
35 mRNA encoding E1, E2, E6 or E7 gene of an HPV. These

antisense oligomers interfere with and/or prevent expression of their target mRNA and thus, may be used for treatment and/or diagnosis of HPV infections as well as for research purposes.

5 Accordingly, provided are oligomers complementary to a target sequence which is a portion of a HPV mRNA or pre-mRNA which encodes E1, E2, E6 or E7. Preferably, the target sequence is in the region of the initiation codon, more preferably from about -25 to about +35
10 relative to the initiation codon where the initiation codon is +1 to +3.

Definitions

As used herein, the following terms have the following meanings unless expressly stated to the
15 contrary.

The term "purine" or "purine base" includes not only the naturally occurring adenine and guanine bases, but also modifications of those bases such as bases substituted at the 8-position, or guanine analogs
20 modified at the 6-position or the analog of adenine, 2-amino purine, as well as analogs of purines having carbon replacing nitrogen at the 9-position such as the 9-deaza purine derivatives and other purine analogs.

The term "pyrimidine" or "pyrimidine base", includes
25 not only the naturally occurring cytosine, uracil and thymine but also modifications to these bases such as 5-propynyluracil, 5-heteroaryluracils and analogs of pyrimidines such as reported heteroaromatic moieties.

The term "nucleoside" includes a nucleosidyl unit
30 and is used interchangeably therewith, and refers to a subunit of a nucleic acid which comprises a 5-carbon sugar and a nitrogen-containing base. The term includes not only those nucleosidyl units having A, G, C, T and U as their bases, but also analogs and modified forms of
35 the naturally-occurring bases, including the pyrimidine-

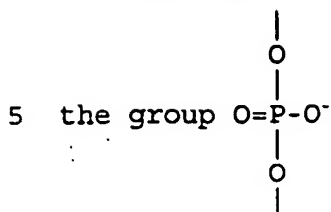
analogs such as pseudoisocytosine and pseudouracil and other modified bases (such as 8-substituted purines). In RNA, the 5-carbon sugar is ribose; in DNA, it is a 2'-deoxyribose. The term nucleoside also includes other
 5 analogs of such subunits, including those which have modified sugars such as 2'-O-alkyl ribose.

10 The term "phosphonate" refers to the group $X=P-R$



wherein X is oxygen or sulfur, R is hydrogen or an alkyl
 15 or aryl group, and thus includes various example of phosphonate and phosphonothioate internucleosidyl linkages. Suitable alkyl or aryl groups include those which do not sterically hinder the phosphonate linkage or interact with each other. The phosphonate group may
 20 exist in either an "R" or an "S" configuration. Phosphonate groups may be used as internucleosidyl linkages (or links) to connect nucleosidyl unit or a nucleosidyl unit and a non-nucleosidy monomeric unit. The term "lower alkylphosphonate" refers to groups where
 25 X is oxygen and R is lower alkyl of 1 to 3 carbon atoms. "Methylphosphonate" refers to groups where X is oxygen and R is methyl. The term "phosphonothioate" refers to those groups where X is sulfur. The term "lower alkylphosphonothioate" refers to groups where X is
 30 sulfur and R is lower alkyl of 1 to 3 carbon atoms. The term "methylphosphonothioate" refers to a phosphonothioate group wherein R is methyl.

The term "phosphodiester" or "diester" refers to



wherein phosphodiester groups may be used as
10 internucleosidyl phosphorus group linkages (or links) to connect nucleosidyl units.

A "non-nucleoside monomeric unit" refers to a monomeric unit wherein the base, the sugar and/or the phosphorus backbone has been replaced by other chemical
15 moieties.

A "nucleoside/non-nucleoside polymer" refers to a polymer comprised of nucleoside and non-nucleoside monomeric units.

The term "oligonucleoside" or "Oligomer" refers to a
20 chain of nucleosides which are linked by internucleoside linkages which is generally from about 4 to about 100 nucleosides in length, but which may be greater than about 100 nucleosides in length. They are usually synthesized from nucleoside monomers, but may also be
25 obtained by enzymatic means. Thus, the term "Oligomer" refers to a chain of oligonucleosides which have internucleosidyl linkages linking the nucleoside monomers and, thus, includes oligonucleotides, nonionic oligonucleoside alkyl- and aryl-phosphonate analogs,
30 alkyl- and aryl-phosphonothioates, phosphorothioate or phosphorodithioate analogs of oligonucleotides, phosphoramidate analogs of oligonucleotides, neutral phosphate ester oligonucleoside analogs, such as phosphotriesters and other oligonucleoside analogs and
35 modified oligonucleosides, and also includes nucleoside/non-nucleoside polymers. The term also includes nucleoside/non-nucleoside polymers wherein one or more of the phosphorus group linkages between

monomeric units has been replaced by a non-phosphorous linkage such as a formacetal linkage, a thioformacetal linkage, a sulfamate linkage, a carbamate linkage, an amide linkage, a guanidine linkage, a nitroxide linkage, or a substituted hydrazine linkage. It also includes nucleoside/non-nucleoside polymers wherein both the sugar and the phosphorous moiety have been replaced or modified such as morpholino base analogs, or polyamide base analogs. It also includes nucleoside/non-nucleoside polymers wherein the base, the sugar, and the phosphate backbone of the non-nucleoside are either replaced by a non-nucleoside moiety or wherein a non-nucleoside moiety is inserted into the nucleoside/non-nucleoside polymer. Optionally, said non-nucleoside moiety may serve to link other small molecules which may interact with target sequences or alter uptake into target cells.

The term "neutral Oligomer" refers to Oligomers which have nonionic internucleosidyl linkages between nucleoside monomers (i.e., linkages having no positive or negative ionic charge) and include, for example, Oligomers having internucleosidyl linkages such as alkyl- or aryl- phosphonate linkages, alkyl- or aryl-phosphonothioates, neutral phosphate ester linkages such as phosphotriester linkages, especially neutral ethyltriester linkages; and non-phosphorus-containing internucleosidyl linkages, such as sulfamate, morpholino, formacetal, thioformacetal, and carbamate linkages. Optionally, a neutral Oligomer may comprise a conjugate between an oligonucleoside or nucleoside/non-nucleoside polymer and a second molecule which comprises a conjugation partner. Such conjugation partners may comprise intercalators, alkylating agents, binding substances for cell surface receptors, lipophilic agents, nucleic acid modifying groups including photo-

cross-linking agents such as psoralen and groups capable of cleaving a targeted portion of a nucleic acid, and the like. Such conjugation partners may further enhance the uptake of the Oligomer, modify the interaction of the Oligomer with the target sequence, or alter the pharmacokinetic distribution of the Oligomer. The essential requirement is that the oligonucleoside or nucleoside/non-nucleoside polymer that the Oligomer conjugate comprises be substantially neutral.

10 The term "substantially neutral" in referring to an Oligomer refers to those Oligomers in which at least about 80 percent of the internucleosidyl linkages between the nucleoside monomers are nonionic linkages.

The term "acid resistant" refers to Oligomers which are resistant, in comparison to deoxyribooligonucleotides, to acid-catalyzed depurination by hydrolysis of the N-glycosyl bond.

20 The term "Triplex Oligomer Pair" refers to first and second Oligomers which are optionally covalently linked at one or more sites and which are complementary to and are capable of hydrogen bonding to a segment of a single stranded target nucleic acid, such as RNA or DNA, and, thus, together with the single stranded target nucleic acid, are capable of forming a triple helix structure therewith.

25 The term "Third Strand Oligomer" refers to Oligomers which are capable of hybridizing to a segment of a double stranded nucleic acid, such as a DNA duplex, an RNA duplex or a DNA-RNA duplex, and forming a triple helix structure therewith.

30 The term "complementary," when referring to a Triplex Oligomer Pair (or first and second Oligomers) or to a Third Strand Oligomer, refers to Oligomers having base sequences which are capable of forming or recognizing hydrogen bonds (and base pairing or

hybridizing) with the base sequence of the nucleic acid to form a triple helix structure.

The term "substantially complementary" refers to Oligomers, including Triplex Oligomer Pairs or Third Strand Oligomers which may lack a complement for each nucleoside in the target sequence, have sufficient binding affinity for the target sequence to form a stable duplex or triple helix complex, as the case may be, and thereby specifically recognize the target sequence and selectively inhibit or down-regulate its expression.

The term "triplet" or "triad" refers a hydrogen bonded complex of the bases of three nucleosides between a base (if single stranded) or bases (if double stranded) of a target sequence, a base of a Second Strand and a Third Strand (if a single stranded target sequence) or a base of a Third Strand (if a double-stranded target).

"MP(Rp)" refers to a methylphosphonate internucleosidyl linkage of Rp chirality.

"MPS" refers to a methylphosphonothioate internucleosidyl linkage.

"MPS(Rp)" refers to a methylphosphonothioate internucleosidyl linkage of Rp chirality.

An oligomer having "alternating MP(Rp)/DE internucleosidyl linkages" refers to an Oligomer wherein methylphosphonate linkages of Rp chirality alternate with phosphodiester linkages ("DE").

An oligomer having "alternating MP(Rp)/PS internucleosidyl linkages" refers to an oligomer wherein methylphosphonate linkages of Rp chirality alternate with phosphorothioate linkages ("PS").

An oligomer having "alternating MPS(Rp)/DE internucleosidyl linkages" refers to an oligomer wherein methylphosphonothioate linkages of Rp chirality alternate with phosphodiester linkages.

An oligomer having "alternating MPS(Rp)/PS internucleosidyl linkages" refers to an oligomer wherein methylphosphonothioate linkages of Rp chirality alternate with phosphorothioate linkages.

5 A "MP(Rp)/DE dimer synthon" refers to a dinucleoside wherein the two nucleosides are linked by a methylphosphonate internucleosidyl linkage of Rp chirality and one of the nucleosides has a 5'- or 3'- coupling group which when coupled to a 3'-OH or a 5'-OH, 10 of another nucleoside or an oligomer will result in a phosphodiester internucleosidyl linkage.

A "MP(Rp)/PS dimer synthon" refers to a dinucleoside wherein the two nucleosides are linked by a methylphosphonate linkage of Rp chirality and one of the 15 nucleosides has a 5'- or 3'- coupling group which when coupled to a 3'-OH or 5'-OH of another nucleoside or an oligomer will result in a phosphorothioate internucleosidyl linkage.

A "MPS(Rp)/DE dimer synthon" refers to a 20 dinucleoside wherein the two nucleosides are linked by a methylphosphonothioate linkage of Rp chirality and one of the nucleosides has a 5'- or 3'- coupling group which when coupled to a 3'-OH or 5'-OH of another nucleoside or an oligomer will result in a phosphodiester 25 internucleosidyl linkage.

A "MP(Rp)/PS₂ dimer synthon" refers to a dinucleoside wherein the two nucleosides are linked by a methylphosphonate linkage of Rp chirality and one of the nucleosides has a 5'- or 3'- coupling group which when 30 coupled to a 3'-OH or 5'-OH of another nucleoside or an oligomer will result in a phosphorothioate internucleosidyl linkage.

A "2'-O-methyl MP(Rp)/2'-O-methyl DE dimer synthon" refers to a dinucleoside wherein two 2'-O-methyl 35 nucleosides are linked by a methylphosphonate linkage of Rp chirality and one of the nucleosides has a 5'- or 3'-

coupling group which when coupled to a 3'-OH or 5'-OH of another nucleoside or an oligomer will result in a phosphodiester internucleosidyl linkage.

Brief Description of the Drawings

5 Figures 1A and 1B depict representation the structures of polycistronic E6/E7 mRNA (Figure 1A) and monocistronic E7 mRNA (Figure 1B) found in HPV-6b or HPV-11 condylomas. The mRNA structures shown are according to Smotkin et al. (J. Virol 63, 1441-1447).

10 The E6/E7 and E7 transcripts are depicted as solid lines. The closed circles at the 5' ends represent the putative promoters. The coding regions for E6 and E7 are indicated by open (E6) or shaded (E7) boxes superimposed on the mRNAs. The nucleotide position of

15 the translation initiation codons and termination codons for E6 and E7 are indicated.

 Figures 2A and 2B depict phylogenetic analysis of the secondary structure of HPV E7 mRNA around the translation initiation codon. Figure 2A depicts

20 alignment of HPV sequences around the E6/E7 boundary. Initial sequence alignments were made with Clustal 3 (Higgins et al., CABIOS 5:151-153 (1989)). Further alignments were made by hand. Secondary structural elements were generated by RNAfold 2 (Scientific and

25 Educational Software), and PCfold 4 (Zuker et al., Nucleic Acids Res. 9:133-148 (1981)). Numbers above HPV-11 are the sequence numbering for HPV-11. Numbers in brackets are extra bases between secondary structural element and the AUG. "cons # 1" and "cons # 2" are the

30 conserved bases for this alignment (R = G, A, Y = U, C). Lower case letters indicate bases conserved in the majority of sequences studied. Sequence elements that are helical are shown in boldface. Periods indicate the base at that position is not contained. Figure 2B

35 depicts a schematic representation of the pseudoknot

structure present upstream of the initiation codon of E7 and also a detailed representation of the pseudoknot sequence found in HPV-11 and in HPV-6b.

Figures 3A and 3B depict inhibition of cell-free translation of monocistronic E7 in RNA with RNase H mediated cleavers (Figure 3A) or with steric blockers (Figure 3B). HPV-11 E7 monocistronic mRNA (circles) or E6/E7 polycistronic mRNA (squares) were translated in rabbit reticulocyte lysates in the absence or in the presence of different concentrations of [MP][DE]₅[MP] oligonucleotide 2567-1 [SEQ. ID. NO. 26], (Figure 3A) or 2'OMeRNA oligonucleotide 2644-1 [SEQ. ID. NO. 18] (Figure 3B). Translation reactions were carried out at 37°C. RNase H at 0.04 units/ul was present in the translations run in the presence of oligonucleotide 2567-1. CAT mRNA at 10 nM, was co-translated as negative control in both cases (triangles). The effect of the oligomers on E7 translation was evaluated after immuno-precipitation of the translation reaction with α E7 anti-serum, size fractionation on SDS PAGE and phospho-image analysis. The effect of the oligomer on CAT translation was evaluated after size fraction on SDS PAGE gel on one aliquot of the reaction mixture followed by phospho-image analysis. Results are expressed as percent of protein translation with respect to the value of translation obtained in the absence of oligonucleotides.

Figures 4A and 4B depict inhibition of E7 and E6 in cell-free synthesis with RNase H mediated cleavers: phosphodiester oligomer 2498 [SEQ. ID. NO. 14] (Figure 4A) or methylphosphonate end-capped diester oligomer 2567-1 [SEQ. ID. NO. 26] (Figure 4B). HPV-11 E6/E7 polycistronic mRNA was translated in rabbit reticulocyte lysates in the absence or in the presence of different concentrations of phosphodiester oligonucleotide 2498-1 (Figure 4A) or [MP][DE]₅[MP] oligonucleotide 2567-1,

(Figure 4B). Translation reactions were carried out at 37°C, in the presence of RNase H at 0.04 units/ul. CAT mRNA, at 10 nM, was co-translated as negative control in both cases. The effect of the oligomers on E7 translation (circles) was evaluated after immuno-precipitation of the translation reaction with α E7 anti-serum, size fractionation on SDS PAGE and phospho-image analysis. The effect of the oligomer on E6 translation (squares) or CAT translation (triangles) was evaluated after size fractionation on SDS PAGE gel of one aliquot of the reaction mixture followed by phospho-image analysis. Results are expressed as percent of protein translation with respect to the value of translation obtained for each protein in the absence of oligonucleotides.

Figure 5 depicts inhibition of transient expression of E7 in COS-7 cells with [Rp-MP/DE] [PS], [Rp-MP/DE] oligomer 3256-1 [SEQ. ID. NO. 32]. E7 expression plasmid pcDNA11E7 (5 ug/ml) and different amounts of antisense oligonucleotide were transfected COS-7 cells in the presence of Transfectam™ (Promega). Cells were incubated with transfection mixture for 4 hours, allowed to recover in media plus serum overnight, and labeled with ³⁵S-cysteine for 5 hours before harvesting. Cells were lysed and E7 protein synthesis was evaluated by immuno-precipitation with α E7 serum followed by SDS-PAGE gel fractionation or protein products and phospho-image analysis. Total protein synthesis was analyzed by SDS-PAGE separation of an aliquot of the cell extract, autoradiography and phospho-image quantitation of all the proteins present in each lane. Results are expressed as percentage of protein translation respect to the value of translation obtained in the absence of oligonucleotide.

Figures 6A and 6B depict reduction of E7 mRNA levels in COS-7 cells treated with [Rp-MP/DE] [PS] [Rp-MP/DE]

oligomer 3256 [SEQ. ID. NO. 32]. E7 expression plasmid pcDNA11E7 (5 ug/ml) alone (lane 1) or together with 5 uM of control oligomer 3215-1 [SEQ. ID. NO. 76] (lane 5) or 0.05 uM (lane 2), 0.5 uM (lane 3) or 5 uM (lane 4) of oligomer 3256-3 [SEQ. ID. NO. 32], was transfected into COS-7 cells using Lipofectamine™ (BRL). Twenty four hours after the transfection, intracellular RNA was extracted and RNase protection assays were carried out using an HPV-11 E7 ³²P-labeled RNA probe (Figure 6A) or a GAPDH ³²P-labeled RNA probe (Figure 6B).

Figure 7 depicts inhibition of expression of E6 and E7 proteins in cells by chimeric methylphosphonate oligonucleotide 3256. Expression plasmids encoding E6 and E7 were transfected into COS-7 cells together with oligonucleotide 3256-3 [SEQ. ID. NO. 32] at 5 uM. Cells were metabolically radiolabelled the next day and immune precipitates were prepared and analyzed by PAGE and autoradiography as described in Figure 5.

Oligonucleotide 3256 targets nucleotides 523-542 of HPV-11, corresponding to the 5' end of the E7 ORF and the 3' end of the E6 ORF. Control oligonucleotide 3218 [SEQ. ID. NO. 46] targets E1.

Figure 8 depicts coding potentials of the HPV-11 E-region transcripts. The circular genome is represented in a linear form with the ORFs (open boxes) and their possible functions indicated above. Vertical dashed lines inside each ORF mark the location of the first AUG codon. All viral E-region transcripts are depicted as arrows in the 5'-to-3' direction, with gaps representing introns spliced out of the transcripts and numbers indicating the nucleotide positions of exon boundaries adjacent to splice donors and acceptors. The closed circle at the 5' end of each message represents the proven or putative promoter, and the arrowheads at the 3' ends designate the polyadenylation sites. Coding potentials, as deduced from the cDNA sequences, are

drawn as open boxes superimposed on each mRNA arrow, and the encodes proteins are named at the 3' end of each transcript.

Figures 9A to 9C depict structures of E6/E7 transcripts found in HPV-16 transformed CaSki cells. The mRNA structures shown are according to Smotkin and Wettstein (Proc. Natl. Acad. Sci. USA 83, 4680-4684). Figure 9A depicts E6/E7 mRNA. Figure 9B depicts E6^I/E7 mRNA. Figure 9C depicts E6^{II}/E7 mRNA. The E6/E7, E6^I/E7 and E6^{II}/E7 transcripts are depicted as solid lines. The introns resulting from two alternative splicing events within the E6 ORF are depicted as dashed lines, the closed circles at the 5' ends represent the putative promoters. The coding regions for E6, E6^I, E6^{II} and E7 are indicated by light (E6) or dark (E7) shaded boxes superimposed on the mRNAs. The nucleotide position of the translation initiation codons, termination codons for E6 and E7, and splice donor and acceptor sites within E6 are indicated.

Figure 10 depicts reduction of E7 mRNA levels in HPV transformed CaSki cells treated with [Rp-MP/DE]₆[PS]₆[Rp-MP/DE], oligomer 3678 [SEQ. ID. NO. 99]. CaSki cells grown in monolayers to about 50% confluency were transfected with 0, 0.3, 1 or 3 μ M of oligomer 3678, or with 3 μ M of control oligomer 3268 [SEQ. ID. NO. 102] using Lipofectamine™ (BRL). Sixteen hours after the transfection, intracellular RNA was extracted and RNase protection assays were carried out using an HPV-16 E7 ³²P-labeled RNA probe. The amount of protected E7 or actin mRNA was quantified using a phosphoimager and the percentage of E7 or actin mRNA respect to Lipofectamine-treated controls was calculated.

Figure 11 depicts reduction of E7 mRNA levels in HPV-transformed CaSki cells treated with [Rp-MP/DE]₆[PS]₆[Rp-MP/DE] oligomers 3678 [SEQ. ID. NO. 99], 3679 [SEQ. ID. NO. 100] and 3680 [SEQ. ID. NO. 101], all

them targeted to the translation initiation codon of HPV-16 E7 and with control oligomer 3268 [SEQ. ID. NO. 102]. CaSki cells grown in monolayers to about 50% confluency were treated with 1 μ M of oligomers and Lipofectamine™ (BRL) for sixteen hours. After the transfection, the cells were washed with CaSki cell culture medium and incubated with the same medium under tissue culture conditions for eight hours. Treatments were repeated for three consecutive days. Intracellular RNA was extracted and RNase protection assays were carried out using an HPV-16 E7 ³²P-labeled RNA probe or a GAPDH ³²P-labeled RNA probe. The amount of protected E7 or GAPDH mRNA was quantified using a phosphoimager and the percentage of E7 of GAPDH mRNA respect to Lipofectamine treated controls was calculated.

Detailed Description of the Invention

Preferred Oligomer Target Regions

Preferred target regions include the portion of an mRNA or pre-mRNA which includes the translation initiation codon, a splice donor site, a splice acceptor site, a coding region, a polyadenylation signal, a 3'-untranslated region and a 5'-untranslated region. Preferably the mRNA or pre-mRNA codes for a human papilloma virus gene selected from E1, E2, E6 and E7. Preferred target sites include the splice donor at 847 in HPV-6b and HPV-11. A corresponding splice donor site is found in other HPVs, for example, at 880 in HPV-16, at 929 in HPV-18, at 877 in HPV-31, at 894 in HPV-33, at 982 in HPV-5, at 966 in HPV-33, at 982 in HPV-5, at 966 in HPV-8 and at 827 in HPV-1. Figure 8 depicts coding potentials of HPV-11 E-region transcripts and may be used to select appropriate target regions. Corresponding target regions for other HPVs may conveniently be determined by reference to alignment of HPV sequences such as that set forth in Figure 2A.

Preferred target regions include those from about -20 to about +20 nucleosides of a splice donor site or a splice acceptor site, a polyadenylation signal, or within a 3'-untranslated region or a 5'-untranslated region or that about -25 to +35 nucleotides of initiation codon.

According to an especially preferred aspect, provided are antisense oligomers which are complementary to a target region of an mRNA or pre-mRNA of an HPV which have from about 14 to about 35 nucleosidyl units, preferably from about 18 to about 24 nucleosidyl units, and more preferably from about 20 to 22 nucleosidyl units.

Especially preferred are oligomers complementary to a portion of the mRNA or pre-mRNA encoding the E7 gene, more preferably the target region is in the region of about -25 to about +35 relative to the initiation codon at +1 to +3. We believe that under physiological conditions a portion of the E6/E7 message forms a pseudoknot structure in a portion of the sequence 5' to the E7 initiation codon (see Figures 2A and 2B and Example E). The formation of the pseudoknot structure may be necessary for termination of E6 translation and/or for initiation of E7 protein translation from the polycistronic message (see Figure 1), since a frame-shift event would be necessary. Pseudoknots have been identified in other eukaryotic mRNAs and have been involved in frame-shift events (Draper, D.E., et al., *Current Opinions in Cell Biology*, Vol. 2, pp. 1099-1103 (1990)). The presence of the pseudoknot structure upstream from the translation initiation codon of E7 may reduce the accessibility of these sequences to targeting by antisense oligonucleotides (see Table I). Accordingly, according to a preferred aspect, provided are oligomers complementary to a portion of the E7 mRNA immediately 5'- or

immediately 3' to the pseudoknot region, more preferably immediately 3'- to the pseudoknot region.

Preferred Oligomers

Although the antisense oligomers of the present invention may incorporate any of the variety of internucleosidyl linkages or backbones, in certain instances preferred are oligomers have an RNase H-activating region and a non-RNase H-activating region. Preferably, the RNase H-activating region comprises a segment of at least three consecutive 2'-unsubstituted nucleosides linked by charged internucleosidyl linkages. Preferred charged internucleosidyl linkages include phosphodiester linkages, phosphorodithioate linkages and phosphorothioate linkages. According to one preferred aspect, a mixed charged linkage sequence is used which includes at least two different charged nucleosidyl linkages. The non-RNase H-activating region comprises a segment of at least two linked nucleosidyl units linked by internucleosidyl linkages which do not activate (or serve as a substrate for) RNase H. According to an especially preferred aspect, at least one of the linkages in the non-RNase H-activating region is chirally-selected. Oligomers having an RNase H-activating region are further described in the commonly-assigned and copending United States Patent Application of Lyle J. Arnold, Jr., Mark A. Reynolds and Cristina Giachetti, "Chimeric Oligonucleoside Compounds", Serial No. 08/233,778, filed April 26, 1994, 08/238,177, filed May 4, 1994 and PCT/US94/13387, filed November 16, 1994. The disclosures of these applications are incorporated herein by reference.

The Oligomers provided herein may form a high affinity complex with a target sequence such as a nucleic acid with a high degree of selectivity. In addition, derivatized Oligomers may be used to bind with and then irreversibly modify a target site in a nucleic

acid by cross-linking (psoralens) or cleaving one or both strands (EDTA). By careful selection of a target site for cleavage, one of the strands may be used as a molecular scissors to specifically cleave a selected
5 nucleic acid sequence. Alternatively, the Oligomers of the present invention may include an RNase H activating sequence.

According to one aspect of the present invention, these antisense Oligomers have a sequence which is
10 complementary to a portion of the RNA transcribed from a selected target gene. Although the exact molecular mechanism of inhibition has not been conclusively determined, it has been suggested to result from formation of duplexes between the antisense Oligomer and
15 the RNA transcribed from the target gene. The duplexes so formed may inhibit translation, processing or transport of an mRNA sequence.

According to an alternate aspect of the present invention, interference with or prevention of expression
20 or translation of a selected RNA target sequence may be accomplished by triple helix formation using Oligomers of the present invention as a Triplex Oligomer Pair having sequences selected such that the Oligomers are complementary to and form a triple helix complex with
25 the RNA target sequence and thereby interfere with or prevent expression of the targeted nucleic acid sequence. Such triple strand formation can occur in one of several ways. Basically, two separate or connected Oligomers may form a triple strand with the single
30 stranded RNA. Further descriptions of the use of Oligomers (including Triplex Oligomer Pairs) to prevent or interfere with the expression of a target sequence of double or single stranded nucleic acid by formation of triple helix complexes is described in the copending U.S
35 Patent Applications Serial Nos. 07/388,027, 07/751,813,

07/772,081 and 07/987,746, the disclosures of which are incorporated herein by reference.

As a general matter, the Oligomers employed will have a sequence that is complementary to the sequence of the target nucleic acid. However, absolute complementarity may not be required; in general, any Oligomer having sufficient complementarity to form a stable duplex (or triple helix complex as the case may be) with the target nucleic acid is considered to be suitable. Since stable duplex formation depends on the sequence and length of the hybridizing Oligomer and the degree of complementarity between the antisense Oligomer and the target sequence, the system can tolerate less fidelity (complementarity) when longer Oligomers are used. This is also true with Oligomers which form triple helix complexes. However, Oligomers of about 8 to about 40 nucleosidyl units in length which have sufficient complementarity to form a duplex or triple helix structure having a melting temperature of greater than about 40°C under physiological conditions are particularly suitable for use according to the methods of the present invention.

With respect to single stranded target sequences, we have found that two strands of a methylphosphonate Oligomer having methylphosphonate linkages (Second and Third Strands) and one strand of a complementary synthetic RNA Oligomer (First Strand) may form a triple helix complex. According to those experiments, the two methylphosphonate strands bind in a parallel orientation. Experiments described triple helix formation with methylphosphonate Oligomers of random sequence of A and G nucleosides which would not make triple helix complexes according to any of the "classical" triplet motifs.

These triple helix complexes formed by binding a target single stranded RNA and two methylphosphonate

Oligomers show high affinity ($T_m > 50^\circ\text{C}$). Formation of these triple helix complexes has been shown to dramatically inhibit translation at sub-micromolar concentrations.

- 5 The triple helix complexes can be formed using Oligomers containing naturally occurring bases (i.e., A, C, G, T or U). Alternatively, if desired for increased stability, certain stabilizing bases such as 2-amino A (for A) or 5-methyl C may be used in place of the
- 10 corresponding naturally occurring base. These bases may increase stability of the triple helix complex by having increased hydrogen bonding interactions and stacking interactions with other bases. Increased stability may result in increased affinity constants which increase
- 15 potency.

- The Oligomers provided herein may be derivatized to incorporate a nucleic acid reacting or modifying group which can be caused to react with a nucleic acid segment or a target sequence thereof to irreversibly modify,
- 20 degrade or destroy the nucleic acid and thus irreversibly inhibit its functions.

Utility and Administration

- These Oligomers may be used to inactivate or inhibit or alter expression of a particular gene or target
- 25 sequence of the HPV in a living cell, allowing selective inactivation or inhibition or alteration of expression. The target sequence may be DNA or RNA, such as a pre-mRNA or an mRNA. mRNA target sequences include an initiation codon region, a coding region, a
- 30 polyadenylation region, an mRNA cap site or a splice junction. These Oligomers could also be used to permanently inactivate, turn off or destroy genes which produced defective or undesired products or if activated caused undesirable effects.

Since the Oligomers provided herein may form duplexes or triple helix complexes or other forms of stable association with transcribed regions of nucleic acids, these complexes are useful in "antisense" or
5 triple strand therapy. "Antisense" therapy as used herein is a generic term which includes the use of specific binding Oligomers to inactivate undesirable DNA or RNA sequences in vitro or in vivo.

Diseases and other conditions such as those caused
10 by infection which a HPV are characterized by the presence of undesired DNA or RNA, which may be in certain instances single stranded and in other instances in double stranded. These diseases and conditions can be treated using the principles of antisense therapy as
15 is generally understood in the art. Antisense therapy includes targeting a specific DNA or RNA target sequence through complementarity or through any other specific binding means, in the case of the present invention by formation of duplexes or triple helix complexes.

20 The Oligomers for use in the instant invention may be administered singly, or combinations of Oligomers may be administered for adjacent or distant targets or for combined effects of antisense mechanisms with the foregoing general mechanisms.

25 In therapeutic applications, the Oligomers can be formulated for a variety of modes of administration, including oral, topical or localized administration. It may be beneficial to have pharmaceutical formulations containing acid resistant Oligomers that may come in
30 contact with acid conditions during their manufacture or when such formulations may themselves be made acidic, to some extent, in order to more compatible with the conditions prevailing at the site of application, e.g., the acid mantle of the skin. Techniques and
35 formulations generally may be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton,

PA, latest edition. The Oligomer active ingredient is generally combined with a carrier such as a diluent or excipient which may include fillers, extenders, binding, wetting agents, disintegrants, surface-active agents, erodible polymers or lubricants, depending on the nature of the mode of administration and dosage forms. Typical dosage forms include tablets, powders, liquid preparations including suspensions, emulsions and solutions, granules, and capsules.

Certain of the Oligomers of the present invention may be particularly suited for oral administration which may require exposure of the drug to acidic conditions in the stomach for up to about 4 hours under conventional drug delivery conditions and for up to about 12 hours when delivered in a sustained release form. For treatment of certain conditions it may be advantageous to formulate these Oligomers in a sustained release form. U.S. Patent No. 4,839,177 to Colombo et al., the disclosure of which is incorporated herein by reference, describes certain preferred controlled-rate release systems. For oral administration, these Oligomers have 2'-O-alkyl nucleosidyl units; these Oligomers are formulated into conventional as well as delayed release oral administration forms such as capsules, tablets, and liquids.

The Oligomers having 2'-O-alkyl nucleosidyl units may be particularly suited for formulation in preparations for topical administration, since the skin has an acid mantle, formulations including these acid resistant Oligomers may prove advantageous. This also can be advantageous in light of the finding that neutral Oligomers will cross skin and mucous membranes as described in U.S. Patent Application Serial No. 07/707,879 which is incorporated by reference. Also it may be desirable to provide formulations which include

acidic media when using acid-resistant neutral Oligomers.

For topical administration, the Oligomers for use in the invention are formulated into ointments, salves, eye drops, gels, or creams, as is generally known in the art.

Systemic administration can also be by transmucosal or transdermal means, or the compounds can be administered orally. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, bile salts and fusidic acid derivatives for transmucosal administration. In addition, detergents may be used to facilitate permeation. Transmucosal administration may be through use of nasal sprays, for example, as well as formulations suitable for administration by inhalation, or suppositories.

To assist in understanding the present invention, the following examples are included which describe the results of a series of experiments. The following examples relating to this invention should not, of course, be construed in specifically limiting the invention and such variations of the invention, now known or later developed, which would within the purview of one skilled in the art are considered to fall within the scope of the present invention as hereinafter claimed.

ExamplesExample 1Preparation of MP(R_p)/DE and MP(R_p)/MP Dimer Synthons

5 A. Preparation of a (CT) Dimer Having a Chirally Pure
 Methylphosphonate Internucleosidyl Linkage Using
 Solution Phase Chemistry

Into a 2 L roto-evaporator flask was placed 10.0 g
(28 mM) of 3'-tert-butyldimethylsilyl thymidine and 26.1
g (35 mM) of 5'-dimethoxytrityl-N⁴-isobutyryl-3'-methyl-
10 N,N-diisopropylaminophosphoramidite-2'-deoxycytidine.
The solids were dissolved in 500 ml of acetonitrile and
evaporated to dryness under vacuum. This process was
repeated with another 500 ml of acetonitrile and then
the flask was released under argon and stoppered with a
15 rubber septa.

This dry solid foam was then dissolved in 500 ml of
acetonitrile ("ACN"), and with manual stirring, treated
all at once with 404 ml tetrazole (180 mM, 0.45 M
tetrazole in THF). Manual stirring is continued for 30
20 seconds and then the flask is allowed to stand for
another 2.5 minutes, after which time the reaction mix
is treated all at once with 275 ml of an oxidizer
solution (I₂/H₂O/lutidine/THF; 25 g/2.5 ml/100 ml/900
ml). The solution was stirred manually and allowed to
25 stand at room temperature for 15 minutes. The resulting
dark amber solution was then treated with bisulfite (2
g/25 ml H₂O), which upon addition, turned the solution
light amber as it reacted with the excess iodide. The
reaction mix was then concentrated to a thick oil and
30 taken up in ethyl acetate ("EtOAc") (500 ml) and washed
with saturated sodium bicarbonate (2 X 250 ml) and H₂O (2
x 250 ml). The organic phase was dried over MgSO₄,
filtered and concentrated to a light colored solid foam,
which upon further drying yielded 35 grams of crude
35 dimer.

The crude dimer was run on HPLC (reverse phase, Waters C18 bondapak) with a program (ACNMETH) starting with 50% acetonitrile and 0.1 M triethylammonium acetate (TEAA, pH ~ 7.0) which increased to 100% acetonitrile over 20 minutes with a linear gradient. Two major peaks were resolved, one at 4.5 minutes, which is residual lutidine and the other at 14.5 minutes which is the mixture of R_p and S_p diastereomers. The ratio of R_p and S_p was determined quantitatively by taking a 5 mg aliquot of the crude product and dissolving it in 1.5 ml of acetonitrile along with 0.5 ml of tetrabutylammonium fluoride (TBAF, 1 M solution in THF). After standing at room temperature for 10 minutes the sample was run on HPLC. Two new peaks were observed at 6.5 and 7.1 minutes and the later eluting peak was gone. The first new peak, which is believed to be the S_p diastereomer, represented 66% (2/1) of the normalized value for the two peaks. The crude product was also analyzed by the (normal phase silica plate) in 75/25 EtOAc/ CH_2Cl_2 ("75/25") with 5% methanol added. The tlc showed two spots with R_f 's of 0.45 and 0.64, respectively; the faster running product (believed to be the R_p form) was less intense than the slower moving one.

The R_p diastereomer was separated on normal phase silica using a methanol step gradient in 75/25 EtOAc/ CH_2Cl_2 . A 7.5 cm by 60 cm column, was loaded with 700 g of silica (first slurried in 2.5 L of neat 75/25 EtOAc/ CH_2Cl_2). The crude dimer was then dissolved in 75 ml of 75/25 EtOAc/ CH_2Cl_2 and loaded onto the column. The column was started with 1% methanol and increased to 2% and finally 3% where the R_p dimer began to elute. The R_p dimer eluted cleanly over several bed volumes while maintaining 3% methanol in the eluent. The S_p dimer was eluted later with 30% methanol. The R_p dimer yield was 11.0 grams, while the S_p yield was 17.8 grams. HPLC analysis (ACNMETH) was performed on the R_p dimer and one

peak was observed at 14.5 minutes. The tlc (75/25 EtOAc/CH₂Cl₂, 5% methanol) of this product, revealed a single spot product with an R_f of 0.55 which, upon treatment with 10% sulfuric acid in ethanol and heat, was both trityl and sugar positive.

The newly resolved R_p dimer, 11.0 g (0.011 M) was dissolved in 110 ml of ACN and treated all at once at room temperature with 22 ml of TBAF (0.022 M, 1 M in THF). The reaction mixture was allowed to stand overnight at ambient temperature. The next morning the reaction was determined to be complete by tlc (75/25, EtOAc/CH₂Cl₂, with 10% methanol); no starting material was detected but a small amount of 5'-DMT-dT was observed, which runs considerably faster on normal phase silica than the 3'-OH of the dimer. The reaction mixture was concentrated on a rotary evaporator to a thick oil which was then dissolved in CH₂Cl₂ (200 ml) and washed with saturated sodium bicarbonate (2 x 100 ml) and H₂O (2 x 100 ml). The organic phase was dried over MgSO₄, filtered, and concentrated to a light yellow solid foam, which was purified on 100 grams of silica (75/25, EtOAc/CH₂Cl₂, with 5% methanol). The 5'-DMT-dT was removed but an impurity at 13.5 minutes (HPLC, ACNMETH) was detected which was first believed to be unreacted starting material (t-BDMS on) but after additional treatment with TBAF this was found not to be the case. A second column, using 100 g of silica and the same eluent was run and smaller fractions were taken; the column was able to successfully separate the two spots. The pure CT-R_p dimer fractions were pooled and concentrated to yield 5.5 grams of a nearly white solid foam.

B. Preparation of a Chirally Pure (CT) MP(R_p)/DE Dimer Synthon

Into a 100 ml round bottom flask was placed 0.5 g (0.55 mMol) CT-3'-OH dimer (product of Example 1A) which was rendered anhydrous by 3 x 20 ml co-evaporations with pyridine. The flask was released from the vacuum system under argon gas and stoppered with a rubber septa. The compound was redissolved in 10 ml acetonitrile and 200 μ l (1.4 mMol, 2.5 eq) TEA were added. To the resulting mixture at room temperature and with manual stirring, was added in one portion 200 μ l (0.90 mmol, 1.6 eq.) 2'-cyanoethyl-N,N-diisopropylchlorophosphoramidite. The reaction mixture was allowed to sit at room temperature before being analyzed by reverse phase HPLC. The HPLC (Beckman System Gold, C18 bondapak, ACN method Solution A was 50/50 ACN/0.1 M TEAA in water, pH 7 and Solution B was ACN. A gradient of 0 to 100% Solution B was run at a rate of 1 ml/minute over 25 minutes) showed complete conversion of starting material and a crude purity of greater than 90 percent. The diastereomers of the phosphoramidite were not resolved. The reaction mixture was concentrated under vacuum to a light yell solid foam. The foam was purified immediately by chromatography on 20 g of normal flash grade silica equilibrated with 5/1/5 ethyl acetate/ acetonitrile/methylene chloride with 2% TEA to give 0.5 g (82% yield) of the above-identified product as an off-white solid foam having a purity of 99.3% as determined by HPLC.

C. Preparation of a Chirally Pure (CT) MP(R_p)/MP Dimer Synthon

The CT-3'-OH dimer, 5.5 g (6 mM), prepared as described in part A above, was rendered anhydrous with two co-evaporations with pyridine. The resulting solid foam was released from the rotary evaporator with argon and stoppered with a rubber septa. The solid foam was

dissolved in 100 ml of 9/1, ACN/CH₂Cl₂, then treated with 1.7 ml triethylamine (TEA, 12 mM). With magnetic stirring, the reaction mix was treated dropwise at room temperature with 1.5 ml chloromethyl-N,N-

5 diisopropylamino phosphine (Cl-MAP, 8 mM). The reaction was monitored on HPLC (ACNMETH) and after 1.5 hours was complete, showing two main products, one at 3.5 minutes which was pyridine and a second at 14.3 minutes which was the desired amidite.

10 The reaction mixture was concentrated on a rotary evaporator using a partial vacuum; the flask which contained the resulting light amber sludge was released under argon and capped. The crude product was immediately passed through a flash column containing 60
15 grams of silica (first equilibrated in 1/1/1 ACN/EtOAc/CH₂Cl₂ with 3% TEA). The product was eluted quickly with this eluent and all U.V. positive fractions were pooled and concentrated. The resulting solid foam was co-evaporated with ACN to remove any residual TEA,
20 then dried overnight under full vacuum. The final product, an off white solid foam, weight 5.0 grams.

Example 2

Preparation of (CU) 2'-O-Methyl MP(R_p)/2'-O-Methyl DE and 2'-O-Methyl MP(R_p)/2'-O-Methyl MP Dimer Synthons

25 A. Preparation of 2'-O-Methyl C Monomer

A 5.0 g (8 mmol) portion of 2'-O methyl cytidine was rendered anhydrous with pyridine co-evaporations (3 X 25 ml) and then dissolved in 50 ml acetonitrile. The solution was treated with 1.65 ml triethylamine ("TEA")
30 (12 mmol, 1.5 eq.) and cooled in an ice bath. The solution was then treated with dropwise addition of 1.65 ml chloromethyl-N,N-diisopropylamino phosphine ("Cl-MAP") over two minutes. The ice bath was removed and the reaction mixture stirred for two hours. The
35 reaction mixture (reaction was determined to be complete

by HPLC) was concentrated to dryness. The residue was dissolved in 20 ml ethyl acetate/heptane (1:1) with 4% TEA, then loaded onto 40 g silica gel equilibrated with the same solvent system. All UV absorbing eluent from
5 the column was collected and pooled, then concentrated to give 5.5 g of the above-identified product (yield about 90%).

B. Preparation of Silyl-Protected 2'-O-Methyl Uridine

Into a 250 ml round bottom flask was placed 5.0 g
10 (9.0 mmol) 5'-DMT, 2'-O-methyl uridine which was rendered anhydrous with dimethylformamide (DMF) co-evaporations (3 X 25 ml). The resulting dry foam was taken up in 50 ml DMF, then treated all at once with 2.4 g (35 mmol, 3.9 eq.) imidazole, followed by dropwise addition of 3.0
15 ml (12 mmol, 1.3 eq.) t-butyldiphenylsilyl chloride. The reaction mixture was stirred at room temperature overnight.

The progress of the reaction was checked by HPLC (ACN method (Solution A was 50/50 ACN/0.1 M TEAA in
20 water, pH 7 and Solution B was ACN; a gradient of 0 to 100% Solution B was run at a rate of 1 ml/minute over 25 minutes) and thin layer chromatography ("TLC") using 5% methanol in methylene chloride, and determined to be complete (no starting material was evident). The
25 reaction mixture was then poured into ice water and taken up in methylene chloride, then washed several times with aqueous sodium bicarbonate and water. The organic phase was dried over magnesium sulfate, filtered and then concentrated to give 7.2 g of a solid foam
30 which gave a single spot on TLC. The solid foam was then dissolved in 70 ml methylene chloride and treated (with rapid magnetic stirring) all at once with 70 ml benzene sulfonic acid, 2% by weight in 2:1 methylene chloride/methanol. After stirring for 15 minutes at
35 room temperature, the reaction mixture was quenched with

10 ml TEA. The resulting detritylated compound was stripped down to a thick amber oil which was then loaded onto 150 g. silica gel equilibrated in heat methylene chloride. The product was eluted from the column using
5 2% methanol (in methylene chloride). After drying, 3.51 g of the above identified product were obtained (yield about 80%).

C. Preparation of (CU) 2'-O-Methyl MP(R_p)/2'-O-Methyl DE Dimer

10 The silyl-protected 2'-O-methyl uridine monomer (product of Example 2B) (3.0 g, 6 mmol) was taken up in 30 ml anhydrous ACN. The 2'-O methyl cytidine amidite monomer (product of Example 2A) (5.5g, 7 mmol, 1.2 eq.) separately, was taken up in 55 ml ACN. Both solutions
15 were allowed to stand over 3 Å molecular sieves overnight at room temperature.

The two solutions were carefully decanted into a single flask and treated with 94 ml tetrazole (0.45 M in ACN, 42 mmol, 7 eq). The resulting mixture was stirred
20 for 4 minutes and then oxidized by addition of 1.5 ml (1.2 eq.) cumene hydroperoxide. The reaction mixture was concentrated to dryness, then taken up in methylene chloride and washed with aqueous sodium bicarbonate and water. The organic phase was dried over magnesium
25 sulfate, filtered and concentrated to give 7.5 g. of a solid foam. The diastereomeric ratio as determined by HPLC by comparison of areas under peaks was 57/43 S_p to R_p.

The R_p diastereomer was isolated by column
30 chromatography using two silica columns (100:1, silica to crude product, equilibrated in 3:1 ethylacetate/methyl chloride with an increasing methanol gradient from 1 to 5%). A total of 1.07 g of pure R_p dimer was isolated.

D. Deprotection of (CU) 2'-O-Methyl Dimer

A 1.07 g (0.90 mmol) portion of the 2'-O methyl CU dimer (product of Example 2C) was dissolved in 10 ml THF and treated all at once with 1.5 ml (1 m in THF, 1.5 eq.) tetrabutylammonium fluoride ("TBAF"). The reaction mixture was stirred at room temperature of r 30 minutes after which time HPLC revealed complete deprotection of the silyl group had been achieved. The reaction mixture was concentrated and the concentrate purified on 10 g silica gel, eluting with 3:1 ethyl acetate/methylene chloride with 5% methanol. The clean fractions were concentrated to give 550 mg of the above-identified pure 5'-OH dimer.

E. Preparation of a Chirally Pure (CU) 2'-O-Methyl (MP/DE) Dimer Synthon

A 230 mg portion of 2'-O-methyl CU 3'-OH dimer (product of Example 2D) was rendered anhydrous by 2 X 5 ml co-evaporations in ACN. The resulting dry solid foam was dissolved in 2.5 ml ACN and then 73 μ l (2.5 eq.) triethylamine ("TEA") and 94 μ l (2.0 eq.) 2'-cyanoethyl-N,N-diisopropyl chlorophosphoramidite (β CNE) were added. The reaction mixture was stirred at room temperature for 2 hours at which time HPLC analysis determined the reaction to be complete. The reaction mixture was dissolved in eluent (3/1/1 ethylacetate/acetonitrile/methylene chloride with 4% TEA) and loaded onto 2 g silica gel equilibrated with 3/1/1 ethylacetate/acetonitrile/methylene chloride with 4% TEA. The column was run using 3/1/1 ethylacetate/acetonitrile/methylene chloride with 1% TEA. The clean fractions, 3 to 25, were concentrated, redissolved in acetonitrile and concentrated again to a solid foam. The foam was dried overnight under full vacuum to give 200 mg of the above-identified product.

F. Preparation of Chirally Pure (CU) 2'-O-Methyl
MP(R₁)/2'-O-Methyl MP Dimer Synthon

Into a 100 ml round bottom flask was placed 400 mg (0.372 mmole) of 2'-O methyl CU dimer (product of Example 2D); it was rendered anhydrous by 1 X 5 ml co-evaporation with acetonitrile. The dry foam was then released from the vacuum system under argon gas, dissolved in 4 ml ACN and stoppered with a rubber septa. The solution was treated with 2 equivalents TEA (103 μ l, 0.744 mmol), followed by 1.75 equivalents chloro-methyl-N,N-diisopropyl phosphine ("Cl-MAP") (118 μ l, 0.651 mmol). The reaction mixture was stirred for 1 hour at room temperature, after which time HPLC showed about 50/50 starting material/product. An additional 50 μ l TEA and 70 μ l Cl-MAP were then added and the mixture stirred for an hour. When HPLC showed only 80% conversion, an additional 30 μ l TEA and 30 μ l Cl-MAP were added and the resulting mixture stirred another hour. At this time HPLC revealed 6% starting material. The reaction mixture was concentrated to dryness. The residue was dissolved in 500 ml 3/1/3 ethylacetate/acetonitrile/methylene chloride with 4% TEA and loaded onto 5 g silica equilibrated in the same solvent system. Fractions were collected. The early fractions were contaminated with a yellow impurity and, thus, were pooled and concentrated separately. The product from those fractions was then repurified by chromatography using the same conditions and pooled with the clean product isolated from the first column. The combined products were co-evaporated with ACN (3 X 5 ml) and dried overnight under full vacuum to give 350 mg (77% yield) of the above identified product which HPLC showed to be 95.5% pure.

Example 3Preparation of 2'-O-Methyl MPS(R_p)/ 2'-O-Methyl-DE and 2'-O-Methyl MPS(R_p)/ 2'-O-Methyl-MP Dimer Synthons

These dimer synthons are prepared by following the
5 procedures described in Example 2, except that in
Paragraph C, an equivalent amount of 3H-1,2-
benzodithiole-3-one, 1,1-dioxide (Beaucage reagent) is
substituted for cumene hydroperoxide. The procedures of
Paragraphs 2E and 2F, respectively, lead to the
10 phosphodiester and methylphosphothioate linkage
combinations.

Example 4Preparation of MPS(R_p)/DE Dimer Synthons

These dimer synthons are prepared by following the
15 procedures of Example 1, except in Paragraph A, an
equivalent amount 3-H-1,2-benzodithiole-3-one, 1,1-
dioxide (Beaucage reagent) is substituted for the
oxidizer solution (I₂/H₂O/lutidine/THF).

Example 520 Preparation of MP(R_p)/PS2 Dimer Synthons

The MP(R_p)/PS2 dimer synthons are prepared as
follows. Isometrically pure R_p dinucleosides having a
free 3'-OH are prepared according to the methods
described in Example 1A. The dinucleoside is converted
25 to the corresponding thiophosphoramidite using
procedures such as those of Plotto et al. (Plotto et al,
Tetrahedron 47:2449-61 (1991)) or Gorenstein et al.,
U.S. Patent No. 5,218,088. The dinucleoside is co-
evaporated three times with anhydrous pyridine, followed
30 by three co-evaporations with toluene. A portion of
dinucleoside (10 mmoles) is dissolved in 200 ml
anhydrous dichloromethane, then three equivalents of
anhydrous diisopropylethylamine followed by 1.5
equivalents of chloro-N,N-diisopropylamino-

thiomethoxyphosphine are added at 0°C with stirring. The reaction is monitored by TLC until determined to be complete.

The product is worked up and purified using the procedures of Example 1B for isolation of the MP(R_p)/DE phosphoramidite.

Example 6

Preparation of MPS(R_p)/PS2 Dimer Synthons

The MPS(R_p)/PS2 dimer synthons are prepared as follows. The isometrically pure R_p dinucleoside with a free 3'-OH is prepared according to the methods of Example 4. Using the dinucleoside, the dimer synthon is prepared by the methods of Example 5.

Example 7

15 Preparation of MPS(R_p)/2'-O Methyl DE Dimer Synthons

The MPS(R_p)/2'-O-methyl DE dimer synthons are prepared using procedures analogous to those of Examples 1 and 3 but using the appropriate protected 2'-deoxynucleoside and protected 2'-O-methyl nucleosides.

20 Example 8

Preparation of a Poly-CT Oligomer Having Alternating MP(R_p)/DE Internucleosidyl Linkages

An oligomer having the sequence 5'-(C*T)-(C*T)-(C*T)-(C*T)-(C*T)-(C*T)-A-3' was prepared using a C*T MP(R_p)/DE dimer synthon prepared according to Example 1. The grouped dinucleosides indicate where the stereochemistry is fixed as the fast eluting isomer on silica gel (putative R_p) and the asterisks indicate the chirally pure linkages.

30 Manual couplings were used to synthesize the oligomer to conserve reagent, although the process can be done on an automated DNA synthesizer. The sequence

was synthesized from the 3'-terminus starting with methacrylate support bound deoxyadenosine.

The protected dinucleoside methylphosphonamidite (22 mg each per required coupling) freshly co-evaporated with pyridine and toluene to ensure dryness were placed into dried 1 ml glass autosampler vials and dissolved in anhydrous acetonitrile to a concentration of 0.1 M (200 μ l per coupling). The vessels were purged with argon and tightly sealed with screw caps with teflon septa.

10 A 1 μ mole scale DNA synthesis column (Milligen) was filled with 1 μ mole of methacrylate support bound deoxyadenosine. The column was attached to a ring stand in a vertical orientation. A male-male luer fitting was attached to the bottom along with an 18 gauge needle to control the effluent. The column was washed with 10 ml acetonitrile using a syringe. The support bound nucleoside was detritylated by passing 3 ml of 2% dichloroacetic acid in dichloromethane through the column over 1.5 minutes. The orange, dimethoxytrityl cation bearing solution was reserved. The column was washed twice with 10 ml each of anhydrous acetonitrile.

The first coupling was accomplished as follows: 10 ml more anhydrous acetonitrile was passed through the column. Then, 200 μ l of the CT methylphosphonamidite was drawn into a 1 ml syringe. Next, 200 μ l of 0.45 M tetrazole in anhydrous acetonitrile was likewise drawn into the syringe containing the methylphosphonamidite. The reagents were rapidly mixed in the syringe, then slowly passed through the column dropwise over three minutes, being sure to lightly draw the plunger up and down to ensure adequate mixing with the support. After 3 minutes, 1 ml of the oxidizing reagent (0.1 M I_2 in 73% tetrahydrofuran, 25% 2,6-lutidine and 2% water) was passed through the column over one minute. The column was washed with 20 ml acetonitrile and then treated with 600 μ l of a solution containing 20% (v/v) acetic

anhydride, 30% (v/v) acetonitrile, 50% (v/v) pyridine and 0.312% (w/v) dimethylaminopyridine. The column was then washed with 20 ml acetonitrile.

The above-described synthetic cycle was repeated
5 until the synthesis was completed. The overall coupling efficiency based on dimethoxytrityl absorbance was 95.7%, for an average of 99.3% per coupling.

The oligomer was then cleaved from the support and deprotected. The support bound oligomer was removed
10 from the synthesis cartridge and placed in a glass 1 dram vial with a screw top. The support was treated for 30 minutes at room temperature with 1 ml of a solution of acetonitrile/ethanol/ NH_4OH (9/9/1). Then, 1 ml of ethylenediamine was added to the reaction vessel and the
15 reaction allowed to sit for 6 hours at ambient temperature in order to go to completion. The supernatant containing the oligomer was then removed from the support and the support was rinsed twice with 2 ml of 1/1 acetonitrile/water; the washings were combined
20 with the supernatant. The combined solution was diluted to 30 ml total volume with water and neutralized with approximately 4 ml of 6 N HCL. The neutralized solution was desalted using a Waters C-18 Sep-Pak cartridge which was pre-equilibrated with 10 ml acetonitrile, 10 ml of
25 50% acetonitrile/100 mM triethylammonium bicarbonate, and 10 ml of 25 mM triethylammonium bicarbonate, sequentially. After the reaction solution was passed through the column, it was washed with 30 ml of water. The product was then eluted with 5 ml of 1/1
30 acetonitrile/water.

The oligomer was purified on HPLC using a Beckman Ultrasphere-reverse phase 4.5 X 250 mm column with an increasing gradient of acetonitrile in 0.5 M triethylammonium acetate (0% to 40% over 40 minutes).
35 The isolated yield was 41 OD_{260} units (35%). The

compound was characterized by electron spray mass spectrometry (calc. 4391/found 4391).

Alternatively, the above-identified oligomer can be synthesized on an automated DNA synthesizer. In this case the appropriate dimer synthons (as used above in the manual synthesis) are dissolved in acetonitrile to a concentration of 0.1 M as described above. The amidite solutions are placed in conical vessels on a Millipore Expedite DNA Synthesizer. All other reagents (oxidizer, deblock, capping reagents and activator) are prepared as described above for the manual synthesis, and applied to the appropriate positions on the instrument as instructed in the manual. Programming parameters for one synthesis cycle are as given in U.S. Patent Application Serial No. 08/158,014. The deprotection and purification of the oligomer is carried out as described above for the manually synthesized oligomer.

Example 9

Preparation of a Poly-CU Oligomer Having Alternating 2'-O-Methyl MP(R_p)/2'-O-Methyl DE and 2'-O-Methyl MP(R_p)/2'-O-Methyl DE Internucleosidyl Linkages

An oligomer having the sequence 5' (C'U) - (C'U) - (C'U) - (C'U) - (C'U) - (C'U) - (C'U) - (C'U) - A-3' was prepared using 2'-O-methyl MP(R_p)/2'-O-methyl DE dimer synthons prepared according to Example 2 hereinabove.

The appropriate dimer synthons were dissolved in acetonitrile to a concentration of 0.1 M. All other reagents used were as described in Example 8.

A 1 μ mole scale DNA synthesis column (Millipore) was filled with 1 μ mole of methacrylate support bound deoxyadenosine. The dimer synthons were coupled sequentially from the 3'-terminus as described in Example 8 except that the coupling time was extended to two minutes. The overall coupling efficiency based on dimethoxytrityl absorbance was 50%, for an average of

91% per coupling. The dimethoxytrityl group was removed from the oligomer at the end of the synthesis.

The deprotection was carried out as described in Example 8. The crude yield was 103 OD₂₆₀ units. The
5 oligomer was purified on HPLC with a Beckman
Ultrasphere-R_p using an increasing gradient of
acetonitrile in 0.5 M triethylammonium acetate (10% to
30% over 30 minutes). The isolated yield was 39 OD₂₆₀
units (38%). The compound was characterized by electron
10 spray mass spectrometry (calc. 4713/found 4712).

This oligomer can also be synthesized on an
automated DNA synthesizer as follows. The appropriate
dimer synthons (as used above in the manual synthesis
are dissolved in acetonitrile as described in Example 8.
15 The amidite solutions are placed in conical vessels on
the Millipore Expedite DNA synthesizer. All other
reagents (oxidizer, deblock, capping reagents and
activator) are prepared as described in Example 8, and
are applied to the appropriate positions on the
20 instrument as instructed by the manual. The same
coupling program as described in Example 8 is used
except that the coupling time is extended to 2 minutes.

The deprotection is carried out as described in
Example 8. The oligomer can be purified on HPLC using
25 as described above for the manual synthesis.

Using similar procedures as described in detail in
Example 8 of U.S. Patent Application Serial No.
08/154,013, the oligomer 5'-(C*U)-(C*U)-(C*U)-(C*U)-
(C*U)-(C*U)-(C*U)-A-3' having 2'-O-methyl MP(R_p)/2'-O-
30 methyl MP (racemic) mixed linkages was prepared. The
product was also characterized by electron spray mass
spectroscopy (calc. 4699.5/found 4701). Automated
synthesis may also be employed as explained above.

Example 10Preparation of 5'-(T'A)-(G'C)-(T'T)-(C'C)-(T'T)-(A'G)-(C'T)-(C'C)-(T'G)-C-3' Having Repeated MP(R_p)/MP Linkage Structures

5 The grouped dinucleosides indicate coupled dimers and the asterisk indicates where the stereochemistry is fixed (chirally defined or chirally pure) as the fast eluting isomer on silica gel (identified as R_p).

 An oligomer having this sequence was synthesized
10 using the appropriate protected dinucleotide methylphosphonamidites prepared using methods such as those described in Examples 1A and 1C above. Manual couplings were used to synthesize the oligomer to conserve reagent, although the process can be done on an
15 automated DNA synthesizer from the 3' terminus starting with support-bound cytidine.

 Each of the desired protected dinucleotide methylphosphonamidites (22 mg each per required coupling), T'A, G'C, T'T (2x), C'C (2x), A'G, C'T, and T'G,
20 freshly co-evaporated with pyridine and toluene to ensure dryness, was placed into a dried 1 ml glass autosampler vial and dissolved with anhydrous acetonitrile to give a concentration of 0.1 M (200 µl were used per coupling). The vials were purged with
25 argon and tightly sealed with screw caps with teflon septa.

 A 1 µmole scale Milligen DNA synthesis column was filled with 1 µmole of support bound cytidine. The column was attached to a ring stand in a vertical
30 orientation. A male-male leur fitting was attached to the bottom along with an 18 gauge needle to control the effluent. The column was washed with 10 ml of ACN using a syringe. The support bound nucleoside was then detritylated by passing 3 ml of 2% dichloroacetic acid
35 in dichloromethane through the column over 1.5 minutes. The orange, dimethoxytrityl cation bearing solution was

reserved. The column was washed twice with 10 ml each of ACN (anhydrous).

The first coupling was accomplished by passing 10 ml more ACN (anhydrous) through the column. Then, 200 μ l of the TG methylphosphonamidite was drawn into a 1 ml syringe. Next, 200 μ L of 0.45 M tetrazole in anhydrous ACN was likewise drawn into the syringe containing the methylphosphonamidite. The reagents were rapidly mixed in the syringe, then slowly passed through the column dropwise over 3 minutes, being sure to lightly draw the plunger up and down to ensure adequate mixing with the support. After 3 minutes, 1 ml of the oxidizing reagent (0.1 M I_2 in 74.25% THF, 25% 2,6-lutidine, and 0.25% water) as passed through the column over 1 minute. The column was then washed with 20 ml of ACN. The column was then treated for 1 minute with 600 μ l of a solution containing 20% (v/v) acetic anhydride, 30% (v/v) ACN, 50% (v/v) pyridine, and 0.312% (w/v) dimethylaminopyridine. The column was washed with 20 ml of ACN.

The synthetic cycle was then repeated with each dinucleotide methylphosphonamidite until the synthesis was completed. The order of addition of dimers after the initial T'G coupling was C'C, C'T, A'G, T'T, C'C, T'T, G'C, and T'A.

The dimethoxytrityl group was removed from the oligomer at the end of the synthesis.

The oligomer was then cleaved from the support and deprotected. The support bound oligomer was removed from the synthesis cartridge and placed in a glass 1 dram vial with a screw top. The support was treated for 30 minutes at room temperature with 1 ml of a solution of acetonitrile/ethanol/ NH_4OH (9/9/1). Then, 1 ml of ethylenediamine was added to the reaction vessel and the reaction mixture allowed to sit for 6 hours at ambient temperature in order to go to completion. The

supernatant containing the oligomer was then removed from the support and the support was rinsed twice with 1 ml of 1/1 acetonitrile/water; the washings were combined with the supernatant. The combined solution was diluted to 50 ml total volume with water and neutralized with approximately 1.7 ml of glacial acetic acid. The neutralized solution was desalted using a Waters C-18 Sep-Pak cartridge which was pre-equilibrated with 5 ml acetonitrile, 5 ml of 50% acetonitrile/water, and 5 ml of water, sequentially. After the reaction solution was passed through the column, it was washed with 50 ml of water. The product was then eluted with 2 ml of 1/1 acetonitrile/water.

The oligomer was purified by HPLC on a reverse phase column (Poros II R/H 4.6 x 100 mm) using a gradient of acetonitrile in water.

Coupling efficiencies are set forth in the table below.

Coupling Efficiencies of
Dinucleotide Methylphosphonamidites

20

25

Dinucleotide	Coupling Efficiency
T'G	99.7%
C'C	90.2%
C'T	91.8%
A'G	85.5%
T'T	97.8%
C'C	83.6%
T'T	100%
G'C	86.2%
T'A	92.4%

Example 11Preparation of 5' - (G*T) - (C*T) - (T*C) - (C*A) - (T*G) - (C*A) - (T*G) - (T*T) - (G*T) - C-3' Having Repeated MP(R_p)/MP Linkage Structures

5 The grouped dinucleotides indicate coupled dimers and the asterisk indicates where the stereochemistry is fixed.

 This sequence was synthesized using the appropriate protected R_p dinucleotide methylphosphonamidites prepared
10 and isolated using procedures such as those described in Examples 1A and 1C above. Manual couplings were used to synthesize the oligomer in order to conserve reagent. However, if desired, the process can be done on an automated DNA synthesizer from the 3' terminus starting
15 with methacrylate support bound 2'-deoxycytidine.

 Each of the desired protected dinucleotide methylphosphonamidites (100 mg), G*T, T*T, T*G, C*A, T*G, C*A, T*C, C*T, and G*T was placed into a dried 3 ml glass conical vial and dissolved with anhydrous acetonitrile
20 to a concentration of 0.1 M. Molecular sieves (3 Å) (0.5 ml volume) were added to each vessel, the vessels purged with argon, and tightly sealed with screw caps with teflon septa. The reagents were allowed to stand overnight prior to use.

25 A 1 μmole scale Milligen DNA synthesis column was filled with 1 μmole of methacrylate support bound 2'-deoxycytidine. The column was attached to a ring stand in a vertical orientation. A male-male luer fitting was attached to the bottom along with an 18 gauge needle to control the effluent. The column was washed with 10 ml
30 of ACN using a syringe. The support bound nucleoside was then detritylated by passing 3 ml of 2.5% dichloroacetic acid in dichloromethane through the column over 3.0 minutes. The orange, dimethoxytrityl cation bearing solution was reserved. The column was
35 washed twice with 10 ml each of ACN (anhydrous).

The first coupling was accomplished by passing 10 ml more ACN (anhydrous) through the column. Then 200 μ l of the G'T methylphosphoramidite was drawn into a 1 ml syringe. Next, 200 μ l of 0.45 M tetrazole in anhydrous
5 ACN was likewise drawn into the syringe containing the methylphosphonamidite. The reagents were rapidly mixed in the syringe, then slowly passed through the column dropwise over 1 minute, being sure to lightly draw the plunger up and down to ensure adequate mixing with the
10 support. After 3 minutes, 1 ml of the oxidizing reagent (0.1 M I_2 in 74.25% THF, 25% 2,6-lutidine, and 0.25% water) was passed through the column over 1 minute. The column was then washed with 20 ml of ACN. The column was then treated for 1 minute with 600 μ l of a solution
15 containing 20% (v/v) acetic anhydride, 30% (v/v) ACN, 50% (v/v) pyridine, and 0.312% (w/v) dimethylaminopyridine. The column was washed with 20 ml of ACN.

The synthetic cycle was then repeated with each
20 dinucleotide methylphosphonamidite until the synthesis was completed. The order of addition of dimers after the initial G'T coupling was T'T, T'G, C'A, T'G, C'A, T'C, C'T and G'T.

The dimethoxytrityl group was removed from the
25 oligomer at the end of the synthesis.

The oligomer was then cleaved from the support and deprotected. The support bound oligomer was removed from the synthesis cartridge and placed in a glass 1 dram vial with a screw top. The support was treated for
30 30 minutes at room temperature with 1 ml of a solution of acetonitrile/ethanol/ NH_4OH (9/9/1). Then, 1 ml of ethylenediamine was added to the reaction vessel and the reaction allowed 6 hours to go to completion. The supernatant containing the oligomer was then removed
35 from the support and the support was rinsed twice with 1 ml of 1/1 acetonitrile/water; the washings were combined

with the supernatant. The combined solution was diluted to 30 ml total volume with water and neutralized with approximately 1.7 ml of glacial acetic acid. The neutralized solution was desalted using a Waters C-18
5 Sep-Pak cartridge which was pre-equilibrated with 5 ml acetonitrile, 5 ml of 50% acetonitrile/water, and 5 ml of water, sequentially. After the reaction solution was passed through the column it was washed with 5 ml of water. The product was then eluted with 2 ml of 1/1
10 acetonitrile/water.

The oligomer was purified by HPLC on a reverse phase column (Poros II R/H 4.6 x 100 mm) using a gradient of acetonitrile in water.

Example 12

15 Preparation of 5' - (G*A) - (G*G) - (A*G) - (G*A) - (G*G) - (A*G) - (G*A) - (A*G) - G-3' Having Repeated MP(R_p)/MP Linkage Structures

The grouped dinucleosides indicate the coupled dimers and the asterisks indicates where the
20 stereochemistry is fixed (chirally defined or chirally pure) as the fast eluting dimer isomer on silica gel (identified as R_p).

This oligomer was prepared using automated synthesis coupling G*A, G*G and A*G MP(R_p)/MP dimer synthons
25 prepared according to the procedures of Examples 1A and 1C.

An amount of G*A, G*G and A*G dimer synthons was dissolved in acetonitrile to give a concentration of 0.1 M and stored over 3 Å molecular sieves (Millipore,
30 Milford, MA) overnight.

The dissolved dimers, with molecular sieves, were placed in conical vessels on a Millipore Expedite DNA Synthesizer which as equipped with end-line filters to remove particulates. All other reagents (oxidizer,
35 deblock, capping reagents and activator) were prepared

and applied to the appropriate positions on the instrument as instructed in the manual. The coupling program was modified to place the oxidizing step immediately subsequent to the coupling step in order to
5 reduce backbone cleavage prior to oxidation. (See Hogrefe, R.I., et al. "An Improved Method for the Synthesis and Deprotection of Methylphosphonate Oligonucleotides" in Methods in Molecular Biology, vol. 20: Protocols for Oligonucleotides and Analogs (ed. 10 Agrawal, S.) pages 143-164, Humana Press, Totowa N.Y. (1983). The programming parameters for one synthesis cycle ("Syn4all-1 μ mol") are set forth in I of U.S. Patent Application Serial No. 08/154,013.

A 1 μ mole scale DNA synthesis column (Millipore) was
15 filled with 1 μ mol of methacrylate support-bound deoxyguanosine and was placed on the DNA synthesizer. The dimers were coupled sequentially from the 3' terminus. The dimethoxytrityl protecting group was removed from the oligomer at the end of the synthesis.
20 The oligomer was then cleaved from the support and deprotected. The support bound oligomer was removed from the synthesis cartridge and placed in a glass 1 dram vial with a screw top. The support was treated for 30 minutes at room temperature with 1 ml of a solution
25 of acetonitrile/ethanol/ NH_4OH (9/9/1). Then, 1 ml of ethylenediamine was added to the reaction vessel and the reaction allowed 6 hours to go to completion. The supernatant containing the oligomer was then removed from the support and the support rinsed twice with 1 ml
30 of 1/1 acetonitrile/water, when combined with the supernatant. The combined solution was diluted to 50 ml total volume with water and neutralized with approximately 1.7 ml of glacial acetic acid. The neutralized solution was desalted using a Waters C-18
35 Sep-Pak cartridge which was pre-equilibrated with 5 ml acetonitrile, 5 ml of 50% acetonitrile/water, and 5 ml

of water, sequentially. After the reaction solution was passed through the column, it was washed with 5 ml of water. The product was then eluted with 1.8 ml of 1/1 acetonitrile/water.

- 5 The crude yield was 87 OD₂₆₀ units. The Oligomers was purified on HPLC using a β -cyclobond standard phase 4.5 X 250 mm column (Azetec, Inc. Whippany, NJ) with a decreasing gradient (80% to 40%) of acetonitrile in 0.05 M triethylammonium acetate (pH 7). The isolated yield
10 was 22 OD₂₆₀ units (25%). The product was characterized by electron spray mass spectrometry (calc. 5407/found 5401).

Example 13

Preparation of an Oligomer Having Alternating MP(R_p)/PS

15 Internucleosidyl Linkages

- An oligomer having alternating MP(R_p)/PS internucleosidyl linkages is prepared using dimer synthons. All the parameters of the synthesis, deprotection and purification are as described in
20 Example 8, except that the oxidizing reagent is replaced by a 0.1 M solution of 3H-1,2-benzodithiole-3-one, 1,1-dioxide or a 0.1 M solution of sulfur in 1/1 carbon disulfide/diisopropylethylamine.

Example 14

25 Preparation of an Oligomer Having Alternating MPS(R_p)/DE Internucleosidyl Linkages

- An oligomer having alternating MPS(R_p)/DE internucleosidyl linkages is prepared using the dimer synthons of Example 4. All other parameters of
30 synthesis, deprotection and purification are as described in Example 8.

Example 15Preparation of an Oligomer Having Alternating MPS(R_p)/PS Internucleosidyl Linkages

An oligomer having alternating MPS(R_p)/PS
5 internucleosidyl linkages is prepared using the dimer
synthons of Example 4. All of the parameters of
synthesis, deprotection and purification are as
described in Example 8, except that the oxidizing
reagent is replaced by a 0.1 M solution of 3H-1,2-benzo-
10 dithiole-3-one, 1,1-dioxide or a 0.1 M solution of
sulfur in 1/1 carbon disulfide/diisopropylethylamine.

Example 16Preparation of an Oligomer Having Alternating MP(R_p)/PS2 Internucleosidyl Linkages

15 An oligomer having alternating MP(R_p)/PS2
internucleosidyl linkages is prepared using the dimer
synthons of Example 5. All of the parameters of
synthesis, deprotection and purification are as
described in Example 15.

20 Example 17Preparation of an Oligomer Having Alternating MPS(R_p)/PS2 Internucleosidyl Linkages

An oligomer having alternating MPS(R_p)/PS2
internucleosidyl linkages is prepared using the dimer
25 synthons of Example 6. All of the parameters of
synthesis, deprotection and purification are as
described in Example 16.

Example 17A30 Preparation of an Oligomer Having Alternating MP(R_p)/2'-O-Methyl DE Internucleosidyl Linkages

An oligomer having alternating MP(R_p)/2'-O-Methyl DE
internucleosidyl linkages is prepared using dimer
synthons similar to those of Example 7. All other

parameters of synthesis, deprotection and purification are as described in Example 9.

Example 18

Preparation of an Oligomer Having Alternating MP(R_p)/MPS

5 Internucleosidyl Linkages

- The preparation of an oligomer having alternating MP(R_p)/MPS internucleosidyl linkages is accomplished using dimer synthons prepared according to Examples 1A and 1C and dissolved and stored over molecular sieves.
- 10 The oxidizing reagent is a 0.1 M solution of 3H-1,2-benzodithiole-3-one, 1,1-dioxide ("Beaucage Reagent", See, Iyer, R.P. et al., JACS 112:1254-1255 (1990)) or a 0.1 M solution of sulfur in 1/1 carbon disulfide/diisopropylethylamine, with synthesis proceeding
- 15 generally as described in Example 12.

Example 19

Preparation of an Oligomer Having 2'-O-Methyl

Nucleosidyl Units and Alternating MP(R_p)/MPS

Internucleosidyl Linkages

- 20 This oligomer is prepared using the dimer synthons as described in Examples 2A-2D and 2F and following the general synthetic procedures of Example 8 of U.S. Patent Application Serial No. 08/154,013, except that the oxidizing reagent described therein is a 0.1M solution
- 25 of 3H-1,2-benzodithiole-3-one, 1,1-dioxide or a 0.1 M solution on 1/1 carbon disulfide/diisopropylamine.

Example 20

Preparation of an Oligomer Having 2'-O-Methyl

Nucleosidyl Units and Alternating MPS(R_p)/MP

30 Internucleosidyl Linkages

This oligomer is prepared using dimer synthons as described in Example 3 above and by following the

parameters of synthesis, deprotection and purification of Example 19.

Example 21

Preparation of an Oligomer Having Alternating MPS(R₁)/MP 5 Internucleosidyl Linkages

This oligomer is prepared using dimer synthons prepared according to Examples 1A and 1C, substituting Beaucage reagent for the oxidizer in Example 1A, and by following the parameters of synthesis, deprotection and
10 purification as described above in Example 12.

Example 22

Preparation of an Oligomer Having Alternating MPS(R₁)/MPS Internucleosidyl Linkages

This oligomer is prepared using dimer synthons as
15 referred to in Example 21 and by following the parameters of synthesis, deprotection and purification as described above in Example 12, except that the oxidizing reagent used therein is replaced by a 0.1 M solution of 3H-1,2-benzodithiole, 1,1-dioxide or a 0.1 M
20 solution of sulfur in 1/1 carbon disulfide/diisopropylethylamine.

Example 23

Preparation of 2'-F Dimer Synthons

Dimer synthons useful in the preparation of the
25 oligomers of the present invention may be prepared using 2'-fluoronucleosides. Methods for preparation of 2'-fluoronucleosides have been reported and are known to those skilled in the art. (See, e.g.: Codington, JOC Vol. 29 (1964) (2'-F U); Mangel, Angew. Chem. 96:557-558
30 (1978) and Doen, JOC 32:1462-1471 (1967) (2'-F C); Ikehara, Chem. Pharm. Bull. 29:1034-1038 (1981) (2'-F G); Ikehara, J. Carbohydrates, Nucleosides, Nucleotides

7:131-140 (1980) (2'-F A), and also Krug, A, Nucleosides & Nucleotides 8:1473-1483 (1989).

The preparation of dimer synthons using 2'-fluoronucleosides may be accomplishing using the
5 procedures analogous to those described for the 2'-O-methyl dimer synthons (See, e.g., Examples 2, 3, and 7). The resulting dimer synthons may be used to prepare oligomers using methods analogous to the methods used for the 2'-O-methyl dimer synthons such as in Example 9.

10 Example 24

Preparation of $MP(R_p)/MP(R_p)/DE$ and $MP(R_p)/MP(R_p)/MP$
Trimer Synthons

The above-identified trimer synthons are prepared using the $MP(R_p)/MP$ dimer synthons of Example 1C. The
15 dimer synthon is coupled to a 5'-hydroxy, 3'-silylated nucleoside according to the methods of Example 1A for the coupling of the 3'-nucleoside to the monomer phosphoramidite.

The selected 5'-hydroxy, 3'-silylated nucleoside (1
20 equivalent) and isomerically pure R_p dimer methylphosphonamide (1.25 equivalents) are weighed into a round bottom flask and dried by co-evaporation with acetonitrile. The resulting foam is dissolved in acetonitrile and treated with a solution of 0.45 M
25 tetrazole in acetonitrile (4.5 equivalents). After 3 minutes, the reaction mixture is oxidized and the reaction product is worked up as described in Example 1A. The diastereoisomers of the 3'-silylated trimer are resolved on a silica gel column as described in Example
30 1A for resolution of the dimer isomers. The configuration of the separated diastereoisomers is determined using 2-D nmr (ROSEY). The trimer having the desired chiral configuration (R_p/R_p) of the two internucleosidyl linkages is converted to a trimer
35 synthon by reaction with chloro- β -cyanoethoxy-N,N-

diisopropylaminophosphoramidite using methods as described in Example 1B. The trimer synthon is worked up and purified using methods as described in Example 1B to achieve the $MP(R_p)/MP(R_p)/DE$ trimer.

- 5 Using similar procedures, an $MP(R_p)/MP(R_p)/MP$ phosphoramidite synthon may be obtained by using chloromethyl-N,N-diisopropylaminophosphine in the final reaction as described in Example 1C for the corresponding dimer synthon. Workup and purification
10 are as described in Example 1C.

Example 25

Preparation of 2'-O-Allyl Dimer and Trimer Synthons and Their Use in Oligomer Synthesis

- The dimer and trimer synthons described, for
15 example, in Examples 1 and 24 can be prepared using 2'-O-allyl nucleosides. The preparation of 2'-O-allyl nucleosides and their use in the preparation of oligomers has been reported (see e.g. Iribarren, et al. (1990) Proc. Natl. Acad. Sci. (USA) 87:7747-51; and
20 Lesnik et al. (1983), Biochemistry 32:7832-8), and such substituted nucleosides are commercially available. The nucleosides are used to prepare dimer and trimer synthons using procedures described hereinabove. The synthons are used to prepare oligomers using methods
25 such as those described in Examples 10, 11, 12, 13 and others above.

Example 26

Preparation of an Oligomer Having $MP(R_p)/MP/DE$ Internucleosidyl Linkages

- 30 The above-identified oligomer is prepared using the trimer synthons of Example 24, or by those in Example 20 of U.S. Patent Application Serial No. 08/154,014, and by following the methods described in Example 8, substituting the trimer synthons for dimer synthons.

All other parameters of synthesis, deprotection and purification are as described in Example 8.

Example 27

Preparation of an Oligomer Having MP(R_p)/MP(R_p)/MP

5 Internucleosidyl Linkages

The above-identified oligomer is prepared using the procedures described in Example 14 of U.S. Patent Application Serial No. 08/154,013.

Example 28

10 Preparation of Oligoribonucleosides

Oligoribonucleotides used in the present examples may be synthesized using general procedures such as described below.

The appropriate 5'-O-dimethoxytrityl-2'-O-tert-
15 butyldimethylsilyl-3'-O-N,N-diisopropyl-β-cyanoethylphosphoramidite nucleosides (Millipore, Hilford, MA) were used for synthesis. Syntheses were done on a 1 μmole scale with a Milligen 8750 automated DNA synthesizer using standard Milligen phosphoramidite
20 procedures with the exception that the coupling times were extended to 12 minutes to allow adequate time for the more sterically hindered 2'-O-tert-butyldimethylsilyl RNA monomers to react. The syntheses were begun on control-pore glass bound 2'-O-tert-
25 butyldimethylsilyl ribonucleosides purchased from Millipore. All other oligonucleotide synthesis reagents were as described in Millipore's standard protocols.

After synthesis, the oligonucleotides were handled under sterile, RNase-free conditions. Water was
30 sterilized by overnight treatment with 0.5% diethylpyrocarbonate followed by autoclaving. All glassware was baked for at least 4 hours at 300°C.

The oligonucleotides were deprotected and cleaved from the support by first treating the support bound

oligomer with 3/1 ammonium hydroxide/ethanol for 15 hours at 55°C. The supernatant, which contained the oligonucleotide, was then decanted and evaporated to dryness. The resultant residue was then treated with
5 0.6 mL of 1 M tetrabutylammonium fluoride in tetrahydrofuran (which contained 5% or less water) for 24 hours at room temperature. The reaction was quenched by the addition of 0.6 mL of aqueous 2 M triethylammonium acetate, pH 7. Desalting of the
10 reaction mixture was accomplished by passing the solution through a Bio-Rad 10DG column using sterile water. The desalted oligonucleotide was then dried.

Purification of the oligoribonucleotides was carried out by polyacrylamide gel electrophoresis (PAGE)
15 containing 15% 19/1 polyacrylamide/bis-acrylamide and 7 M urea using standard procedures (See Maniatis, T. et al., Molecular Cloning: A Laboratory Manual, pages 184-185 (Cold Spring Harbor 1982)). The gels were 20 cm wide by 40 cm long and 6 mm in width. The
20 oligoribonucleotides (60 OD Units) were dissolved in 200 µL of water containing 1.25% bromophenol blue and loaded onto the gel. The gels were run overnight at 300 V. The product bands were visualized by UV backshadowing and excised, and the product eluted with 0.5 M sodium
25 acetate overnight. The product was desalted with a Waters C18 Sep-Pak cartridge using the manufacturer supplied protocol. The product was then ³²P labelled by kinasing and analyzed by PAGE.

Example 29

30 Preparation of Racemic Methylphosphonate Oligonucleotides

Various racemic oligomers were synthesized using 5'-(dimethoxytrityl) deoxynucleoside-3'-[(N,N-diisopropylamino)methyl]-phosphonoamidite monomers. Solid-phase
35 synthesis was performed on methacrylate polymer supports

with a Biosearch Model 8750 DNA synthesizer according to the manufacturer's recommendations except for the following modifications: the monomers were dissolved in acetonitrile at a concentrations of 100 mM, except dG,
5 which was dissolved in 1/1 acetonitrile/dichloromethane at 100 mM. DEBLOCK reagent = 2.5% dichloroacetic acid in dichloromethane. OXIDIZER reagent = 25 g/L iodine in 0.25% water, 25% 2,6-lutidine, 72.5% tetrahydrofuran. CAP A = 10% acetic anhydride in acetonitrile. CAP B =
10 0.625% N,N-dimethylaminopyridine in pyridine.

The dimethoxytrityl group was removed from the oligonucleotide at the end of the synthesis.

The oligonucleotide was then cleaved from the support and deprotected. The support bound
15 oligonucleotide was removed from the synthesis cartridge and placed in a glass 1 dram vial with a screw top. The support was treated for 30 minutes at room temperature with 1 ml of a solution of acetonitrile/ethanol/ NH_4OH (9/9/1). Then, 1 ml of ethylenediamine was added to the
20 reaction vessel and the reaction allowed 6 hours to go to completion. The supernatant containing the oligonucleotide was then removed from the support and the support rinsed twice with 2 ml of 1/1 acetonitrile/water, when combined with the supernatant.
25 The combined solution was diluted to 30 ml total volume with water and neutralized with approximately 4 ml of 6 N HCl. The neutralized solution was desalted using a Waters C-18 Sep-Pak cartridge which was pre-equilibrated with 10 ml acetonitrile, 10 ml of 50% acetonitrile/100
30 mM triethylammonium bicarbonate, and 10 ml of 25 mM triethylammonium bicarbonate, sequentially. After the reaction solution was passed through the column it was washed with 30 ml of water. The product was then eluted with 5 ml of 1/1 acetonitrile/water.

The oligonucleotide was purified by HPLC on a reverse phase column (Whatman RAC II) using a gradient of acetonitrile in 50 mM triethylammonium acetate.

Example 30

5 Chimeric Oligonucleotide Assembly From MP(R_p)/MP and MP(R_p)/DE Dimer Synthons and Phosphoramidite and Methylphosphonamidite Monomer Synthons

MP(R_p)/MP dimer synthons contained a methylphosphoramidite coupling group at the 3' end.
10 When coupled together to make an oligomer, these synthons give racemic methylphosphonate linkages at every other position. R_p-MP/DE dimer synthons contained a β-cyanoethyl phosphoramidite coupling group at the 3'-end. Both types of dimer synthons were synthesized as
15 described in Example 1. Methylphosphonamidite monomer synthons were synthesized at JBL Scientific (San Luis Obispo, CA). Betacyanoethyl phosphoramidite monomer synthons were purchased from Milligen/Bioscience.

All synthons were coupled using a Milligen Expedite™
20 automated DNA synthesizer. The coupling programs for each synthon were as tabulated below. To generate a phosphorothioate bond during a coupling step, the program "Thioate-5μM" was used with either a dimer or monomer synthon containing a β-cyanoethyl
25 phosphoramidite coupling group.

DIESTER -- 5 μ M

```

/* -----
/*      Function      Mode      Amount      Time(sec)      Description
/*                      /Arg1      /Arg2
/* -----
$Deblocking
144 /* Advance Frac */ NA          1          0      "Event out ON"
  0 /* Default      */ WAIT         0         1.5    "Wait"
141 /* Photometer S */ NA          1          1      "START data collection"
 16 /* Dblk         */ PULSE        10          0      "Dblk to column"
 16 /* Dblk         */ PULSE       200         49      "Deblock"
 38 /* Wsh A to C1  */ PULSE        80          0      "Flush system with Wsh A"
141 /* Photometer S */ NA          0          1      "STOP data collection"
 39 /* Gas A to C1  */ PULSE        10          0      "Gas A to C1 waste"
144 /* Advance Frac */ NA          2          0      "Event out OFF"
 12 /* Wsh A        */ PULSE       200          0      "Wsh A"
$Coupling
  1 /* Wsh          */ PULSE        10          0      "Flush system with Wsh"
  2 /* Act          */ PULSE        10          0      "Flush system with Act"
 18 /* A + Act      */ PULSE         5          0      "Monomer + Act to column"
 18 /* A + Act      */ PULSE        18         60      "Couple monomer"
  2 /* Act          */ PULSE         3         10      "Couple monomer"
  1 /* Wsh          */ PULSE         7        56.1    "Couple monomer"
  1 /* Wsh          */ PULSE        50          0      "Flush system with Wsh"
$Capping
 13 /* Caps         */ PULSE        25          0      "Caps to column"
 12 /* Wsh A        */ PULSE        50          0      "Wsh A"
 12 /* Wsh A        */ PULSE       150          0      "End of cycle wash"
$Oxidizing
 15 /* Ox           */ PULSE        50         30      "Ox"
 12 /* Wsh A        */ PULSE        50          0      "Flush system with Wsh A"
$Capping
 13 /* Caps         */ PULSE        25          0      "Caps to column"
 12 /* Wsh A        */ PULSE        50          0      "Wsh A"
 12 /* Wsh A        */ PULSE       150          0      "End of cycle wash"

```

THIOATE -- 5 μ M

```

/* .....
/*      Function      Mode  Amount  Time(sec)  Description
/*      /Arg1      /Arg2
/* .....
SDeblocking
  144 /* Advance Frac */ NA      1      0      "Event out ON"
    0 /* Default      */ WAIT    0      1.5    "Wait"
  141 /* Photometer S */ NA      1      1      "START data collection"
    16 /* Dblk        */ PULSE   10     0      "Dblk to column"
    16 /* Dblk        */ PULSE  200    49      "Deblock"
    38 /* Wsh A to Cl  */ PULSE   80     0      "Flush system with Wsh A"
  141 /* Photometer S */ NA      0      1      "STOP data collection"
    39 /* Gas A to Cl  */ PULSE   10     0      "Gas A to Cl waste"
  144 /* Advance Frac */ NA      2      0      "Event out OFF"
    12 /* Wsh A        */ PULSE  200     0      "Wsh A"
SCoupling
   1 /* Wsh            */ PULSE   10     0      "Flush system with Wsh"
   2 /* Act            */ PULSE   10     0      "Flush system with Act"
  23 /* 6 + Act       */ PULSE    6     0      "Monomer + Act to column"
  23 /* 6 + Act       */ PULSE   17    60      "Couple monomer"
   2 /* Act            */ PULSE    4    10      "Couple monomer"
   1 /* Wsh            */ PULSE    7   55.9    "Couple monomer"
   1 /* Wsh            */ PULSE   50     0      "Flush system with Wsh"
SCapping
  13 /* Caps          */ PULSE   25     0      "Caps to column"
  12 /* Wsh A         */ PULSE   50     0      "Wsh A"
  12 /* Wsh A         */ PULSE  150     0      "End of cycle wash"
SOxidizing
  17 /* Aux           */ PULSE    5     0      "SOx"
  17 /* Aux           */ PULSE   45    60      "SOx"
  12 /* Wsh A         */ PULSE   50     0      "Flush system with Wsh A"
SCapping
  13 /* Caps          */ PULSE   25     0      "Caps to column"
  12 /* Wsh A         */ PULSE   50     0      "Wsh A"
  12 /* Wsh A         */ PULSE  150     0      "End of cycle wash"

```

METHYLPHOSPHONATE -- 5 μ M

/* -----					
/*	Function	Mode	Amount	Time(sec)	Description
/*			/Arg1	/Arg2	
/* -----					
SDeblocking					
144	/* Advance Frac	*/ NA	1	0	"Event out ON"
0	/* Default	*/ WAIT	0	1.5	"Wait"
141	/* Photometer S	*/ NA	1	1	"START data collection"
16	/* Dblk	*/ PULSE	10	0	"Dblk to column"
16	/* Dblk	*/ PULSE	200	49	"Deblock"
38	/* Wsh A to Cl	*/ PULSE	80	0	"Flush system with Wsh A"
141	/* Photometer S	*/ NA	0	1	"STOP data collection"
39	/* Gas A to Cl	*/ PULSE	10	0	"Gas A to Cl waste"
144	/* Advance Frac	*/ NA	2	0	"Event out OFF"
12	/* Wsh A	*/ PULSE	200	0	"Wsh A"
SCoupling					
1	/* Wsh	*/ PULSE	10	0	"Flush system with Wsh"
2	/* Act	*/ PULSE	10	0	"Flush system with Act"
18	/* A + Act	*/ PULSE	5	0	"Monomer + Act to column"
18	/* A + Act	*/ PULSE	18	60	"Couple monomer"
2	/* Act	*/ PULSE	3	10	"Couple monomer"
1	/* Wsh	*/ PULSE	7	56.1	"Couple monomer"
1	/* Wsh	*/ PULSE	50	0	"Flush system with Wsh"
SOxidizing					
15	/* Ox	*/ PULSE	50	30	"Ox"
12	/* Wsh A	*/ PULSE	50	0	"Flush system with Wsh A"
SCapping					
13	/* Caps	*/ PULSE	25	0	"Caps to column"
12	/* Wsh A	*/ PULSE	50	0	"Wsh A"
12	/* Wsh A	*/ PULSE	150	0	"End of cycle wash"

MP (R_p) /MP -- 5 μM

.....						
/*	Function	Mode	Amount	Time(sec)	Description	
/*			/Arg1	/Arg2		
/*					
SDeblocking						
144	/* Advance Frac	*/ NA	1	0	"Event out ON"	
0	/* Default	*/ WAIT	0	1.5	"Wait"	
141	/* Photometer S	*/ NA	1	1	"START data collection"	
16	/* Dblk	*/ PULSE	10	0	"Dblk to column"	
16	/* Dblk	*/ PULSE	200	49	"Deblock"	
38	/* Wsh A to Cl	*/ PULSE	80	0	"Flush system with Wsh A"	
141	/* Photometer S	*/ NA	0	1	"STOP data collection"	
39	/* Gas A to Cl	*/ PULSE	10	0	"Gas A to Cl waste"	
144	/* Advance Frac	*/ NA	2	0	"Event out OFF"	
12	/* Wsh A	*/ PULSE	200	0	"Wsh A"	
SCoupling						
1	/* Wsh	*/ PULSE	10	0	"Flush system with Wsh"	
2	/* Act	*/ PULSE	10	0	"Flush system with Act"	
18	/* A + Act	*/ PULSE	5	0	"Monomer + Act to column"	
18	/* A + Act	*/ PULSE	18	60	"Couple monomer"	
2	/* Act	*/ PULSE	3	10	"Couple monomer"	
1	/* Wsh	*/ PULSE	7	56.1	"Couple monomer"	
1	/* Wsh	*/ PULSE	50	0	"Flush system with Wsh"	
SOxidizing						
15	/* Ox	*/ PULSE	50	30	"Ox"	
12	/* Wsh A	*/ PULSE	50	0	"Flush system with Wsh A"	
SCapping						
13	/* Caps	*/ PULSE	25	0	"Caps to column"	
12	/* Wsh A	*/ PULSE	50	0	"Wsh A"	
12	/* Wsh A	*/ PULSE	150	0	"End of cycle wash"	

MP(R_y)/DE -- 5 μ M

```

/* .....
/*      Function      Mode      Amount      Time(sec)      Description
/*      /Arg1      /Arg2
/* .....
SDeblocking
  144 /* Advance Frac */ NA          1          0      "Event out ON"
    0 /* Default      */ WAIT         0         1.5    "Wait"
  141 /* Photometer S */ NA          1          1      "START data collection"
    16 /* Dblk        */ PULSE        10          0      "Dblk to column"
    16 /* Dblk        */ PULSE       200         49      "Deblock"
    38 /* Wsh A to Cl  */ PULSE        80          0      "Flush system with Wsh A"
  141 /* Photometer S */ NA          0          1      "STOP data collection"
    39 /* Gas A to Cl  */ PULSE        10          0      "Gas A to Cl waste"
  144 /* Advance Frac */ NA          2          0      "Event out OFF"
    12 /* Wsh A        */ PULSE       200          0      "Wsh A"
SCoupling
  1 /* Wsh            */ PULSE        10          0      "Flush system with Wsh"
  2 /* Act            */ PULSE        10          0      "Flush system with Act"
  18 /* A + Act       */ PULSE         5          0      "Monomer + Act to column"
  18 /* A + Act       */ PULSE        18         60      "Couple monomer"
  2 /* Act            */ PULSE         3         10      "Couple monomer"
  1 /* Wsh            */ PULSE         7        56.1    "Couple monomer"
  1 /* Wsh            */ PULSE        50          0      "Flush system with Wsh"
SOxidizing
  17 /* Aux           */ PULSE        50         30      "Aux"
  12 /* Wsh A         */ PULSE        50          0      "Flush system with Wsh A"
SCapping
  13 /* Caps          */ PULSE        25          0      "Caps to column"
  12 /* Wsh A         */ PULSE        50          0      "Wsh A"
  12 /* Wsh A         */ PULSE       150          0      "End of cycle wash"

```

Applying one or more of these coupling routines with the appropriate dimer or monomer synthons, one skilled in the art can recognize that each of the chimeric oligomers described in subsequent examples can be
5 synthesized.

Deprotection and purification of each chimeric oligomer was done essentially as described in Examples 8 through 12.

The identities of certain chimeric oligomers made
10 according to this Example, as well as other compounds, were confirmed by electrospray mass spectrometry as shown in the following table:

Seq. #	Sequence	Backbone	MW Predicted	MW Found
2624-1	3'-CTGTTG TACGT ACCTTCTG-5'	Racemic MP	5725	5726
2571-1	3'-CTGTTG TACGT ACCTTCTG-5'	75%MP(R _h)	5725	5725
3130-3	3'-CCTGTTG TACGT ACCTTCTG-5'	MP(R _h)OE	6028	6029
2566-1	3'-CCTGTTG TACGT ACCTTCTG-5'	PS	6354	6357.9
2567-1	3'-CCTGTTG(TACGT)ACCTTCTG-5'	[MP]OE[MP]	6022	6018
2687-1	3'-CCTGTTG(TACGT)ACCTTCTG-5'	[75%R _h MP]OE[75%R _h MP]	6022	6022
3169-1	3'-CCTGTTG(TACGT)ACCTTCTG-5'	[MP(R _h)OE]OE[MP(R _h)OE]	6033	6034
3214-1	3'-CCTGTTG(TACGT)ACCTTCTG-5'	[MP(R _h)OE][PS]OE[MP(R _h)OE]	6082	6081
3257-1	3'-CCTGTTG(TACGTAC)CTTCTG-5'	[MP(R _h)OE][PS]OE[MP(R _h)OE]	6100	6100
3256-1	3'-CCTGTTG(TACGT)ACCTTCTG-5'	[MP(R _h)OE][PS][MP(R _h)OE]	6113	6114
3258-1	3'-CGTCCTCGATT(CCTTC)GATGGTAC-5'	[MP(R _h)OE][PS]OE[MP(R _h)OE]	7300	7299
3260-1	3'-CGTCCTCGATT(CCTTC)GATGGTAC-5'	[MP(R _h)OE][PS][MP(R _h)OE]	7331	7331
3261-1	3'-CTCTTCTTCTA(GTGAC)CTATATGG-5'	[MP(R _h)OE][PS]OE[MP(R _h)OE]	7313	7310
3262-1	3'-CTCTTCTTCTA(GTGAC)CTATATGG-5'	[MP(R _h)OE][PS][MP(R _h)OE]	7345	7346
3269-1	3'-ACGTCTGATCA(GTAAC)TAACTCAC-5'	[MP(R _h)OE][PS]OE[MP(R _h)OE]	7309	7308
3270-1	3'-ACGTCTGATCA(GTAAC)TAACTCAC-5'	[MP(R _h)OE][PS][MP(R _h)OE]	7341	7340

1. (Parenthesis) refers to the portion that activates RNaseH; the linkage on the 3'-side of the indicated nucleoside is charged.

Example APreparation of Plasmid Expressing a Polycistronic E6/E7 mRNA

An expression vector having an insert coding for
5 HPV11 E6/E7 was prepared using the expression vector
pRc/CMV (Invitrogen) as follows:

The plasmid pRc/CMV was linearized with *Hind* III.
The recessed 3' ends were filled with the 5'-3'
polymerase activity of T_4 DNA polymerase.

10 A full length clone of HPV-11 cloned at the *Bam*H I
Site in pBR322 was digested with the restriction enzymes
Bst EII and *Hinf* I. The 873 base pair fragment
containing the E6 and E7 open reading frames was
purified on agarose gel. The restriction ends of this
15 fragment were modified by filling in the recessed 3'-
ends with T_4 DNA polymerase.

The vector and insert were ligated with T_4 DNA ligase
and transformed into DH5 α *E. Coli*. recombinants were
screened for appropriate insert and orientation as well
20 as E6/E7 transcription and translation activity.

This plasmid (pRc/CMV11-E6/E7) was used to prepare
the polycistronic mRNA used in the cell free translation
system described in Example F.

Example BPreparation of Plasmid Having Monocistronic E7 Insert

An expression vector having an HPV-11 E7 insert was prepared using pcDNA-1 (Invitrogen) as follows.

- 5 The plasmid pcDNA was digested with *Bam*H I and with *Xba* I. A fragment containing the complete open reading frame of HPV-11 (from -30 till the termination codon) flanked by *Bam* HI and *Xba* I restriction sites was prepared by PCR using standard protocols. The digested
- 10 vector and fragment were ligated with T4 DNA ligase and transformed into MC 1061/P3 cells. Recombinants were screened for appropriate insert, transcription and translation.

- This plasmid (pcDNA E7) was used to prepare the
- 15 monocistronic mRNA used in the cell-free translation system described in Example G and in the transient expression assay in COS-7 cells described in Example J and in the RNase H cleavage assay of Example I.

Example C

20 Preparation of Plasmid Having a E1 Insert

An expression vector having an HPV-11 E1 insert was prepared using the expression vector pRc/CMV (Invitrogen) as follows.

- The plasmid pRC/CMV was linearized with *Hind* III.
- 25 The recessed 3' ends were filled in with T₄ DNA polymerase, and then the plasmid was cut with *Xba* I.

- A full length clone of HPV-11 cloned at the *Bam* HI site in pBR322 was digested with the restriction enzyme *Apa*I. The recessed 3'-ends were filled in with the
- 30 5'-3' polymerase activity of the Klenow fragment of DNA polymerase I. The modified DNA was next cut with *Spe* I and a 2428 base pair fragment containing the complete E1 ORF was agarose gel purified.

- The modified vector and E1 insert were ligated with
- 35 T₄ DNA ligase and transformed into DH5 α *E. Coli*.

Recombinants were screened for appropriate insert, transcription and translation.

This plasmid (pRc/CMVII-E1) was used in the cell free translation system of Example M.

5 Example D

Preparation of Plasmid Having an E2 Insert

An expression vector having an HPV-11 E2 insert was prepared using pRc/CMV (Invitrogen) as follows.

10 The plasmid pRc/CMV was linearized with *Hind* III, followed by treatment with calf thymus alkaline phosphatase.

To isolate the E2 open reading frame, a full length clone of HPV-11, cloned at the *Bam* HI site in pBR322 was digested with the restriction enzymes *Xmn* I and *Ssp* I.

15 The recessed 3' ends were filled in with the 5'-3' polymerase activity of the Klenow fragment of DNA polymerase I. *Hind* III linkers were then added. The 1309 base pair fragment containing the complete E2 ORF was agarose gel purified.

20 The modified vector and E2 insert were ligated with T₄ DNA ligase and transformed into DH5 α *E. Coli*. Recombinants were screened for appropriate insert, transcription and translation.

25 This plasmid (pRc/CMVII-E2) was used in the cell-free translation system of Example O.

Example E

Evidence for Pseudoknot Structure Upstream of Translation Initiation Codon of HPV E7

30 Phylogenetic analysis was used to determine the secondary structure of HPV E7 mRNA from N380 to N580 (HPV-11 sequence numbers). Sequence fragments of 13 different HPV types were aligned using a computer algorithm. The alignments were further adjusted by hand to give the final alignment.

The secondary structures generated with this analysis were consistent with only one structure: a pseudoknot just upstream of the translation initiation codon of E7. The pseudoknot found here is a hairpin stem-loop which forms an additional helix using bases in the loop. This structure is best defined by HPV-44, -43, -11, and -6b, all them associated with genital warts. Similar structures with lower levels of stability were also found in the other HPV types analyzed.

Figures 2A and 2B explain the pseudoknot structures for HPV-6b and HPV-11. Please note that the AUG in boldface is the start codon for the E7 mRNA. The pseudoknot starts 6 nucleotides upstream from the from the HPV-11 E7 AUG. In the case of high risk HPVs a variable number of nucleotides is present between the pseudoknot structure and the initiation codon of E7.

It is interesting to note that in low risk HPVs as well as in HPV 2a, the E6 and E7 proteins are encoded by a polycistronic mRNA, and that the reading frames of both proteins overlap each other by about 20 bases (see Figure 1A). Since the reading frame for E7 is -1 (or +2) as compared to E6, this pseudoknot may be involved in the ribosomal frame shifting needed for translation of both proteins, or perhaps in regulation of the level of E6/E7 protein production. Pseudoknots have been shown to have both of these functionalities in other systems. Interestingly, in the case of high risk HPVs where E6 and E7 do not overlap, and in which E7 is generated from a spliced E6/E7 mRNA, a variable number or nucleotides is present between the structure and the initiation codon of E7.

Example FDemonstration of Activity of Antisense OligomersTargeted to HPV-11 E7 in Cell Free Translation Extracts

- Mono-cistronic (100 nM) HPV-11 E7 or polycistronic
- 5 (50 nM) HPV-11 E6/E7 RNA was co-translated with chloramphenicol acetyl transferase (CAT) RNA (2 to 10 nM) in cell-free rabbit reticulocyte extracts (Promega). The contents of each assay system was as follows.

COMPONENT	FINAL CONCENTRATION
In vitro transcribed uncapped RNA	(As noted above)
³⁵ S-cysteine	0.8 mCi/mL
5 Amino acids mixture, cysteine deficient	20μM each
Rabbit reticulocyte lysate	72% by volume
RNasin (Promega)	0.5 units /μL
Oligomer	1 to 10μM

- 10 Cell free translation was performed at 37°C for 60 minutes and was stopped by addition of SDS gel loading buffer and incubation at 95° for 3 minutes.

- Translation of E7 was evaluated after immuno-precipitation with αE7 goat anti-serum and protein A
 15 sepharose, followed by SDS-PAGE and phospho-image analysis. This protocol was used in the cell-free translations of Examples G and L.

Selection of target sequence for low risk HPV E7

- The effect of phosphodiester oligonucleotides 2406-1
 20 [SEQ. ID. NO. 6], 2552-1 [SEQ. ID. NO. 7], 2458-1 [SEQ. ID. NO. 8], 2457-1 [SEQ. ID. NO. 9], 2549-1 [SEQ. ID. NO. 10], 2553-1 [SEQ. ID. NO. 11], 2518-1 [SEQ. ID. NO. 12], 2525-1, [SEQ. ID. NO. 13], 2498-1 [SEQ. ID. NO. 14], 2492-1 [SEQ. ID. NO. 15], 2589-1 [SEQ. ID. NO. 16]
 25 and 2590-1 [SEQ. ID. NO. 17] (see Table I) on cell-free translation of polycistronic HPV-11 E6/E7 mRNA was evaluated using the protocol described above.

- Translations were made in the presence 0.02 units/μl of RNaseH, and oligonucleotide at either 0.01, 0.1 or 1 μM.
 30 CAT mRNA was co-translated as negative control. Please note that all the oligonucleotides tested, but 2589-1

and 2590-1, and 2492-1 are fully complementary to HPV-11 as well as the HPV-6b sequence..

Table I summarizes the results we obtained. The percentages of inhibition were calculated as the difference between the inhibition of E7 protein synthesis and control CAT translation. For example, in cases where no specific inhibition was indicated, no differences between inhibition of E7 and CAT was observed. Overall, the results presented here showed that the specific inhibition of the oligonucleotides increased as the target sequence was located downstream from N542 (AUG-7), indicating that HPV-11 mRNA sequences present downstream from N542 (AUG-7) are accessible to oligonucleotides.

Because oligonucleotide 2498-1 complements HPV-6b as well as HPV-11 and showed good specific translation inhibition of E7, it was selected as our preferable oligomer sequence.

Finally, the results presented here add support to the presence of a pseudoknot just upstream from the translation initiation site of E7 (as described in Example E), since the phosphodiester data shows a decrease in oligomer activity as the oligomer overlaps with the pseudoknot.

Results are reported in Tables I and VI.

Example G

Inhibition of Cell-Free Translation of Monocistronic E7 or Polycistronic E6/E7 mRNA with RNaseH Mediated Cleavers or With Steric Blockers.

Since HPV-11 E7 may be translated from a polycistronic E6/E7 mRNA (Figure 1A) or a monocistronic E7 mRNA (Figure 1B), it was important to compare the antisense activity of oligomers on both mRNAs. In particular, it was of interest to know the ability to inhibit translation of the polystronic mRNA, as compared

to the monocistronic mRNA, by RNase H-mediated cleavers or steric blockers. Oligonucleotide 2567-1 [SEQ. ID. NO. 26], a chimeric methylphosphate oligomer was used as an example of a RNase H-mediated cleaver, and

5 oligonucleotide 2644-1 [SEQ. ID. NO. 18], a 2'-OMeRNA, was used as an example of a steric blocker. HPV-11 E7 monocistronic mRNA (circles) or E6/E7 polycistronic mRNA (squares) were translated in rabbit reticulocyte lysates in the absence or in the presence of different

10 concentrations of [MP] [DE], [MP] oligonucleotide 2567-1, or 2'-OMeRNA oligonucleotide 2644-1, as described in Example F. RNase H at 0.04 units/ μ l was present in the translations run in the presence of oligonucleotide 2567-1, and CAT mRNA, at 10 nM, was co-translated as a

15 negative control (triangles) in both cases.

Analysis of the data presented in Figure 3A indicated that oligonucleotide 2567-1 was able to inhibit E7 translation whether E7 was translated from the monocistronic (circles) or from the polycistronic

20 mRNA (squares). The oligomer was only slightly more active on the monocistronic mRNA than on the polycistronic mRNA. Figure 3B depicts the results obtained with oligonucleotide 2644-1. This result showed that the activity of the steric blocker on either

25 mRNA was lower than the activity observed with the RNase H mediated cleaver 2567-1, and that oligomer 2644-1 produced very little inhibition of E7 synthesis when it is translated from the polycistronic mRNA. In the case of both the oligomers, no effects on CAT translation

30 were observed, indicating that their activity was very specific.

Overall these results indicated that inhibition of HPV E7 translation will be more successfully achieved by the use of RNase H mediated cleavers.

Example HDetermination of Hybridization of Antisense Oligomers to RNA

The melting temperatures (T_m) for RNA duplex forming antisense oligomers were determined at equimolar concentrations (1.2 μ M) of oligomer and synthetic RNA target in 1 ml of buffer containing 20 mM DPO₄, pH 7.2, 0.1 mM NaCl, 0.1 mM EDTA and 0.03% sarkosyl. The reaction mixtures were heated by 80°C and then slowly cooled to 4°C over approximately 4 to 6 hours. The annealed samples were then transferred to 1 cm quartz cuvettes; absorbance at 260 nm as a function of temperature was monitored using a Varian Cary Model 3E Spectrophotometer containing a 6 X 6 temperature-controlled sample holder interfaced with an IBM compatible PC computer. The temperature was varied from 5°C to 80°C at a ramp rate of 1°C/minute. The T_m for each melt profile was defined at the point corresponding to the first derivative (of the A_{260} -temperature function).

Results are reported in Tables VI and IX.

Example IDemonstration of Activity of Antisense Oligomers in a Cell-Free RNase H Cleavage Assay

In vitro transcribed, uncapped mono-cistronic RNA was prepared by transcribing plasmid pCDNA11E7 with RNA polymerase (Ambion MegaScript).

The E7 RNA was incubated at a concentration of 100 nM in the presence of 0.04 units μ L E. Coli. RNase H (Promega), 3.5 mM MgCl₂, 25 mM KCl, 70 mM NaCl and 20 mM potassium acetate at 37°C for 30 minutes. Reactions were stopped by addition of formamide gel loading buffer followed by heating to 100°C for 5 minutes.

Samples were analyzed by 4% Urea-PAGE analysis, followed by staining with ethidium bromide.

Percentages of cleavage of E7 mRNA, in the presence of RNase H, of methylphosphonate chimeric oligomers 2567-1 [SEQ. ID. NO. 26], 3169-1 [SEQ. ID. NO. 29], 3214-1 [SEQ. ID. NO. 30], 3257-1 [SEQ. ID. NO. 31], 3341-1 [SEQ. ID. NO. 33], and 3336-1 [SEQ. ID. NO. 34], are shown in Table XIV. Dose response effects were obtained for all the oligomers at the concentrations tested. The order of potency was 3169-1 \approx 3257-1 > 3214-1 \approx 2567-1 > 3336-1 > 3341-1. All oligomers showed good specificity, cleaving E7 mRNA in one position.

Results are reported in Table XIV.

Example J

Demonstration of Activity of Antisense Oligomers in Transiently Transfected COS-7 Cells

COS-7 cells (obtained from ATCC [Catalog CRL 1651]) were seeded at 1×10^5 cells/well in 24 well plates and then cultured overnight in cell culture media (90% DMEM, 10% fetal bovine serum and 50 I.U./ml penicillin, 50 mg/ml streptomycin and 0.25 μ g/ml amphotericin B). After 24 hours the cells were approximately 80 to 90% confluent.

A transfection cocktail of 2.5 μ g/mL pcDNA1E7, 50 μ g/mL transfectam (Promega) and varying concentrations of oligomer was prepared and incubated for 15 minutes at room temperature after a 2 second vortex mix.

Cells were washed on the plates two times, 1 ml/well with Optimem (Gibco-BRL). Then 0.5 mL transfection cocktail per well was applied to duplicate wells. The plates were incubated for 4 hours in 5% CO₂ at 37°C. After incubation cells were washed two times, 1 mL/well with cell culture media and cultured overnight. Then cells were washed twice, 1 mL/well with cysteine deficient DMEM and then incubated for 30 minutes in cysteine deficient DMEM under cell culture conditions. Cells were labelled by incubation with 250 μ Ci of ³⁵S-

cysteine/well in 500 μ L cysteine deficient DMEM without serum for 5 hours. The cells were then washed twice, 1 mL/well with 1X phosphate buffered saline and then lysed with 100 μ L SDS sample buffer (50 mM Tris-Cl [pH 6.8], 100 nM dithiothreitol, 2% sodium dodecyl sulfate, 0.1% bromophenol blue, 10% glycerol). Wells were washed with 100 μ L RIPA buffer (10 mM Tris-Cl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate) and combined with sample buffer lysate.

E7 synthesis was evaluated by immunoprecipitation of E7 protein with goat anti-HPV-11 E7 serum and protein A sepharose beads (Sigma). Immunoprecipitated E7 protein was quantitated by SDS-PAGE and phospho-image analysis. Total protein synthesis was evaluated by SDS-PAGE and phospho-image analysis of a fraction of the transfected cell lysate before immunoprecipitation.

Total protein synthesis was analyzed by SDS-PAGE separation of an aliquot of the cell extract, autoradiography and phospho-image quantitation of all the proteins present in each lane. Results are expressed as percentage of protein translation respect to the value of translation obtained in the absence of oligonucleotide.

Results of a representative experiment performed with the methylphosphonate chimeric oligomer 3256-1 [SEQ. ID. NO. 32] is shown in Figure 5. In addition, Table VII summarizes the IC50 and IC90 values obtained with chimeric oligomers 3169-1 [SEQ. ID. NO. 29], 3214-1 [SEQ. ID. NO. 30], 3257-1 [SEQ. ID. NO. 31], 3256-1 [SEQ. ID. NO. 32], 3327-1 [SEQ. ID. NO. 77] and 3336-1 [SEQ. ID. NO. 34].

It is clear from this example that chimeric oligonucleotides 3214-1, 3257-1 and 3256-1, which contain all phosphorothioate ([PS]) or alternating phosphorothioate/phosphodiester ([PS/DE]) linkages in

the middle and chiral methylphosphonate dimers linked by phosphodiester linkages ([Rp-MP/DE]) as end-blocks are potent inhibitors of transient expression of HPV E7 protein in COS-7 cells.

- 5 Chimeric oligonucleotides with phosphodiester linkages in the middle, such as 3169-1, were not potent in the cell-based assay, although they proved to be very potent in the cell-free assay (Table VI). This difference may be due to the intracellular instability
10 of the phosphodiester linkage.

Finally, oligonucleotides containing 2'OMe modification in the sugar of the nucleosides present at the ends (oligonucleotides 3327-1 and 3336-1) were less potent than the corresponding chimeras with [Rp-MP/DE]
15 ends.

Results are reported in Table VII and Figure 5.

Example K

Chimeric Methylphosphonate Oligomers Produced Reduction of HPV-11 E7 mRNA Levels in COS-7 Cells

- 20 To prove that the downregulation of E7 protein levels produced by the chimeric oligomers in cells was the result of an antisense interaction with its mRNA (due to a RNase H mediated cleavage of the oligomer-RNA duplex), the intracellular levels of E7 mRNA after
25 treatment of transient transfected cells with a chimeric oligomer were determined using a RNase protection assay. The RNA probe used in the assay complements HPV-11 N564 to N841, so that if E7 mRNA is present, a protected band of 278 nucleotides should be expected. As control for
30 the RNase protection assay, we determined that the Protection was specific for E7 mRNA, as no protection to the probe was rendered by incubation of it with 10 ug of yeast RNA or by incubation with RNA from mock-transfected cells. Moreover, the signal was present
35 after DNase treatment of the samples prior to the RNase

protection assay, denoting protection of the probe due to E7 mRNA rather than to E7 cDNA.

The phosphoimage presented in Figure 6A, shows that treatment of the cells with oligomer 3256 [SEQ. ID. NO. 32] at 0.05, 0.5 or 5 uM produced a dose-dependent decrease in the amount of E7 probe (lanes 1-4), indicating a reduction in the amount of E7 mRNA present in the cell extracts. Treatment of the cells with 5 uM of an oligomer targeted to HPV-11 E2 [SEQ. ID. NO. 76] did not significantly reduce the amount of E7 mRNA present (lane 5), denoting that the reduction in E7 mRNA was sequence-specific. Moreover, probing of the same oligomer-treated cell extracts with a probe targeted to GAPDH mRNA showed no reduction in the probe after the RNase treatment, indicating no effect of oligomer 3256 on this cellular mRNA (Figure 6B). Overall these results confirm that the E7 mRNA reduction produced by oligomer 3256 was specific and demonstrate that the reduction in E7 protein levels is due to a specific interaction of the oligomer with the E7 mRNA.

Example L

Inhibition of HPV-11 E6 as well as HPV-11 E7 Cell-free Synthesis with One Single RNaseH Medicated Cleaver Targeted to the Translation Initiation Codon of E7

As shown in Figure 1A, HPV-11 E6 proteins are translated from the polycistronic E6/E7 mRNA containing the ORF of E6 upstream from the ORF of E7. Since oligomers targeted to the translation initiation codon of E7 will also complement the 3' portion of the ORF of E6, it was important to determine the activity of these oligomers on E6 production.

HPV-11 E6/E7 polycistronic mRNA was translated in rabbit reticulocyte lysates in the absence or in the presence of different concentrations of oligomers. Translation reactions were carried out at 37°C, in the

presence of RNase H at 0.04 units/ul. CAT mRNA, at 10 nM, was co-translated as negative control in both cases. The effect of the oligomers on E7 translation (circles) was evaluated after immuno-precipitation of the translation reaction with α E7 antiserum, size fractionation on SDS PAGE and phospho-image analysis. The effect of the oligomers on E6 translation (squares) or CAT translation (triangles) was evaluated after size fractionation by SDS PAGE of one aliquot of the reaction mixture followed by phospho-image analysis. Results are expressed as percent of protein translation with respect to the value of translation obtained for each protein in the absence of oligonucleotides.

Analysis of the activity of steric blockers or RNase H mediated cleavers indicated that while steric blockers had no significant effect on E6 production, RNase H mediated cleavers significantly inhibited E6 production in the cell-free assay. As an example, Figures 4A and 4B depict the results obtained with phosphodiester oligonucleotide 2498-1, [SEQ. ID. NO. 14] (Figure 4A) and with [MP][DE]₂[MP] oligomer 2567-1, [SEQ. ID. NO. 26] (Figure 4B).

Example M

In vitro Inhibition of HPV-11 E6 Protein Expression With Chimeric Methylphosphonate Oligomers Targeted to the E7 Translation Start Site

HPV-11 E6 is translated from a E6/E7 mRNA containing the E6 ORF upstream from the E7 ORF (Figure 1). Consequently, oligonucleotides that are targeted to the translation initiation codon of E7 (HPV-11 bases 532-542) will also complement the 3' portion of the E6 ORF. Since our previous results indicated that the inhibition of E7 production in cells is due to an interaction between the oligomer and the mRNA (Example K) and that oligomers targeted to E7 diminished the amount of E6

synthesized in a cell-free reaction (Example L), it was important to determine the activity of oligonucleotides targeted to the E7 translation start on E6 production in cells.

- 5 To examine whether chimeric oligonucleotides targeted to E7 could also block expression of E6, plasmids pCDNA11E7, or pED/E6 [plasmid pED/E6 contains the HPV-11 E6 gene plus 32 upstream bases (HPV nucleotides 72-555) behind the adenovirus major late
10 promoter and tripartite leader], encoding either E7 or E6, were transfected into COS-7 cells together with chimeric oligomer 3256 [SEQ. ID. NO. 32] at 5 uM.

- Levels of E6 and E7 protein were evaluated after 18 hours by metabolic labeling and precipitation with
15 specific anti-E7 or anti-E6 serum, followed by autoradiography. As shown in Figure 7, oligomer 3256 inhibited expression of E7 (lane f) as expected. This oligonucleotide also blocked expression of E6 (lane c). A control chimeric oligomer 3218 [SEQ. ID. NO. 46]
20 targeted to the translation start site of the E1 protein of HPV-11 had no effect on expression of E7 (lane g) and little effect on E6 (lane d). These results agree with the cell-free data and indicate that oligomer 3256 in addition to the previous observed inhibition of E7, was
25 able to produced downregulation of E6 protein production.

- It is clear that chimeric oligonucleotides are effective when targeted to either the translation start site of an mRNA (E7 in this case) or to the 3' end of an
30 ORF (E6). This results supports the idea that chimeric oligonucleotides can block synthesis of multiple proteins encoded in polycistronic mRNAs.

Example NDemonstration of Oligomer Activity by Microinjection in VERO Cells(i) Micro injection

5 Oligomers were microinjected together with E2
(pRc/CMV 11-E2) or E7 (pcDNA E7) expression plasmids at
50 $\mu\text{g}/\mu\text{l}$ into the cytoplasm of VERO cells according to
the following procedure. On the day preceding
injection, VERO cells (approximately 2×10^5 cells/ml)
10 were plated on coverslips. Plasmid DNA was diluted in
PBS to a concentration of 20 $\text{ng}/\mu\text{l}$ (E7) or 50 $\text{ng}/\mu\text{l}$ (E2)
in an eppendorf tube. The tubes containing plasmid DNA
were centrifuged for 15 minutes at 1,400 rpm. the tubes
were set on ice prior to microinjection. A 2 μL aliquot
15 of plasmid DNA solution was loaded onto a femto top.
The tip was set with coverslip at 45°C , the pressure on
the microinjector was set at 80 and the injection was
performed. The coverslips were incubated at 37°C
overnight after injection. At 16 hours post-injection,
20 cells were fixed and immunostained with goat anti-E7
polyclonal antibody, as explained below.

(ii) Indirect Fluorescence Immunoassay

Expression level of E2 or E7 was assessed using a
fluorescent antibody assay as follows.

25 Coverslips were fixed in 10% formaldehyde in PBS for
20 minutes at room temperature and then washed twice
with PBS.

Prior to use in this assay, goat anti-HPV-11 E7 or
HPV-11 E2 serum was preabsorbed with VERO cells as
30 follows. Confluent VERO cells from two T-150 flasks
were scraped and then washed twice with PBS. then 200
 μl serum was added to the cell pellet and mixed at 40°C
overnight. the mixture was centrifuged; the supernate
was removed to a new tube. the preabsorbed serum was
35 stored in 50% glycerol at -20°C .

Coverslips were incubated with goat anti-HPV-11 E7 or HPV-11 E2 protein serum preabsorbed as set forth below at a 1:1000 dilution in PBS for two hours at room temperature. Coverslips were washed with PBS three
 5 times, five minutes per wash. Coverslips were incubated with FITC-conjugated Donkey Anti-Goat IgGAb (Jackson, ImmunoResearch, Cat #705-095-147) at 1:200 dilution in PBS. Coverslips were washed with PBS three times and then air-dried. Coverslips were mounted with 50%
 10 glycerol on slide glass. Coverslips were then examined under UV lights.

Results are reported in Tables VI, VII, and XII.

Example O

Demonstration of Activity of Antisense Oligomers

15 Targeted to E2 in Cell-Free Translation Extracts

E2 RNA was prepared by transcribing plasmid pRc/CMV-11E2 with T7 RNA polymerase using an Ambion MegaScript kit, following the manufacturer's directions.

In vitro transcribed E2 mRNA was cell-free
 20 translated in rabbit reticulocyte lysates (Promega). The final concentrations of each component of the assay system was as follows:

25	In vitro transcribed uncapped RNA:	50 nM
	³⁵ S-Methionine:	1.3 uCi/ul
	Potassium Acetate:	20 mM
	Amino acid mixtures, methionine deficient:	50 uM
	Rabbit Reticulocyte Lysate:	33 % vol/vol
	RNAsin:	None or 0.5 units/ul

Cell-free translation was performed at 37°C for one
 30 hour and was stopped by addition of SDS gel loading buffer and incubation at 95°C for 3 minutes. Translation of E2 was evaluated after separation of the translation mix by SDS-PAGE, followed by phospho-image

analysis. To determine the effect of oligomers targeted to the translation initiation codon of E2, *in vitro* transcribed E2 mRNA was translated in the presence of 0.02 or 0.04 units/ μ l of RNase H, and using
5 oligonucleotide concentrations ranging from 0.01 to 10 μ M. CAT mRNA was co-translated, or translated in independent translation reactions as control.

As shown in Tables X and XI, different levels of inhibition of E2 cell-free translation and of
10 specificity regarding the CAT control mRNA were obtained with the oligomers tested. Overall, the results indicated that in most cases, steric blockers were significantly less potent than RNase H activators. (Compare the activity of oligomer 3102-1 [SEQ. ID. NO.
15 52] with 3129-1 [SEQ. ID. NO. 64] or 3124-1 [SEQ. ID. NO. 57]; or the activity of 3103-1 [SEQ. ID. NO. 53] with 3173-1 [SEQ. ID. NO. 55]. In addition, end-blocked chimeric oligomers were more specific than all phosphodiester oligomers (compare the activity and
20 specificity of oligomer 3102-1 [SEQ. ID. NO. 52] with 3233-1 [SEQ. ID. NO. 60] or 3234-1 [SEQ. ID. NO. 61]).

Our results using the cell-free translation assay indicated that the region of the E2 mRNA around the translation initiation codon, from AUG-12 to AUG+16, was
25 accessible to antisense targeting, and that oligonucleotide backbone influenced the degree of antisense activity and specificity of the oligomer.

Example P

Demonstration of Activity of Antisense Oligomers

30 Targeted to E1 in Cell-Free Translation Extracts

E1 mRNA was prepared by transcribing plasmid pRc/CMV-11E1 with T7 RNA polymerase using an Ambion MegaScript kit or an Ambion Message Machine kit, following the manufacturer's directions.

In vitro transcribed E1 mRNA was cell-free translated in wheat germ extracts (Promega). The final concentrations of each component of the assay system was as follows:

5	In vitro transcribed E1 mRNA:	100 nM (uncapped) or 15 nM (capped)
	³⁵ S-Methionine:	1 uCi/ul
	Potassium Acetate:	75 mM
	Amino acid mixtures, methionine deficient:	80 uM each
10	Wheat Germ Extract:	50 % vol/vol
	RNAasin:	0.5 units/ul
	Oligomers:	0.01 to 10 uM

Cell-free translation was performed at room temperature for one hour and was stopped by addition of SDS gel loading buffer and incubation at 95°C for 3 minutes. Translation of E1 was evaluated after separation of the translation mix by SDS-PAGE, followed by phospho-image analysis.

To determine the effect of oligonucleotides targeted to the translation initiation codon of E1 and to the splice donor for E2, E5a, E5b and E1⁺E4 present at N847, in vitro transcribed E1 mRNA was translated in the presence or absence of oligomers 2555-1 [SEQ. ID. NO. 40], 2556-1 [SEQ. ID. NO. 41], 2557-1 [SEQ. ID. NO. 42], 2744-1 [SEQ. ID. NO. 47], 3105-1 [SEQ. ID. NO. 43], 3106-1 [SEQ. ID. NO. 44], 3196-1 [SEQ. ID. NO. 45] and 3218-1 [SEQ. ID. NO. 46] (see Table III). Translations were performed in the presence of 0.02 units/ul of RNase H, and using oligomer concentrations ranging from 0.01 to 10 uM. CAT mRNA was translated in an independent translation reaction as control.

As shown in Table IX, steric blocker oligomers 3105-1 and 3196-1 showed low potency, and high specificity,

while phosphodiester 2555-1, 2556-1, and 2557-1 produced high levels of inhibition of E1 translation at 1 uM, and different levels of non-specific translation inhibition of the CAT control. Among the phosphodiesters, oligomer
5 2557-1 (targeted to the splice donor site at N847) showed less non-specific effects and oligonucleotide 2744-1, a 2'-OMe end-capped diester, was the most potent and specific.

According to these results, the preferred target
10 sequence around the translation initiation codon of E1 was AUG+14 and AUG+33 regarding oligomer backbones, a chimeric backbone is preferable.

Example Q

Demonstration of Activity of Antisense Oligomers

15 Targeted to E6 in Cell-Free Translation Extracts

Polycistronic E6/E7 mRNA was prepared by transcribing the plasmid pRc/CMV11-E6/E7 with T7 RNA polymerase using an Ambion MegaScript kit, following the manufacturer's directions.

20 In vitro transcribed E6/E7 mRNA (50nM) was cell-free translated in rabbit reticulocyte lysates (Promega) as described in Example F. Cell-free translation was performed at 37°C for one hour and was stopped by addition of SDS gel loading buffer and incubation at
25 95°C for 3 minutes. Translation of E6 was evaluated after separation of the translation mix by SDS-PAGE analysis, followed by phospho-image analysis.

To determine the effect of oligomers targeted to the translation initiation codon of E6, in vitro
30 transcribed E6/E7 mRNA was translated in the presence or absence of the oligonucleotides showed in Table V. Translations were performed in the presence of 0.02 or 0.04 units/ul of RNase H, and using oligomer concentrations ranging from 0.01 to 10 uM. CAT mRNA was
35 co-translated as control. As shown in Table XIII,

different levels of inhibition of E6 cell-free translation were obtained with the oligomers tested. In general, low levels of inhibition of E6 translation were obtained, which suggested that the region of the E6 translation initiation codon, from AUG-10 to AUG+21, presented poor accessibility to antisense targeting. The best results were obtained with oligomer 3215-1 [SEQ. ID. NO. 76], a 20-mer chimeric methylphosphonate oligomer targeted to AUG-10.

10 Example R

Downregulation of HPV-16 E7 mRNA levels in HPV-transformed CaSki cells treated with [Rp-MP/DE] [PS] [Rp-MP/DE] oligomer 3678 as compared with a Control [Rp-MP/DE] [PS] [Rp-MP/DE] oligomer

15 CaSki cells (obtained from ATCC, Catalog CRL 1550) were seeded at 4×10^5 cells/60 mm dish and cultured in cell culture medium (90% RPMI, 10% fetal bovine serum, 50 I.U./mL penicillin and 50 mg/mL streptomycin). After 28 hours the cells were approximately 50% confluent.

20 A transfection mix containing 18 μ g/mL of Lipofectamine (BRL) and various concentrations of oligomer was prepared, vortex mixed, and incubated for 15 minutes at room temperature. Cells were washed two times with 4 mL/dish of OptiMem (Gibco BRL), and 2 mL of transfection mix per dish was applied to duplicate samples. The cells were incubated for 16 hours in 5% CO₂ at 37°C. After the transfection the cells were washed two times with 4 mL/well of PBS and total cytoplasmic RNA was extracted using RNAzol™B (TEL-TEST, Inc.)

25 following the manufacturer's directions.

The intracellular level of HPV-16 E7 mRNA after the oligomer treatment was determined using a RNase protection assay. The RNA probe used in the assay complements HPV-16 N566 to N855, so that if E7 mRNA is present, a protected band of 289 nucleotides should be

35

expected. As control for specificity of the oligomer treatment, the intracellular levels of actin mRNA were determined by probing an aliquot of the intracellular RNA with a commercially available probe targeted to
5 actin (Ambion, pTRI- β -actin-125-Human).

Phosphoimage quantitation of the HPV-16 E7 protected RNA bands indicated that treatment of the cells with oligomer 3678 [SEQ. ID. NO. 99] produced a dose-dependent reduction in the amount of E7 mRNA present in
10 the cell extracts (see Figure 10). Maximum inhibition obtained was about 60% when the oligomer was present at 1 to 3 μ M. Treatment of the cells with 3 μ M of control oligomer targeted to the CAT poly(A) site [SEQ. ID. NO. 102] [5'CCTCATTTT(ATTAG)GAAAGGACAG-3'] did not
15 significantly reduce the amount of E7 mRNA present, denoting that the reduction in E7 mRNA was sequence-specific. Moreover, probing of the same oligomer-treated cell extracts with a probe for actin mRNA showed no reduction in the levels of actin in cell extracts
20 treated with 0.3 or 1 μ M of oligomer 3678 [SEQ. ID. NO. 99], indicating specific antisense effect at these oligomer concentrations. Treatment of the cells with 3 μ M of oligomer 3678 [SEQ. ID. NO. 99] or with control oligomer [SEQ. ID. NO. 102] showed about 40% inhibition
25 of actin, indicating non-specific effects at these high concentrations. Overall these results showed that oligomer 3678 [SEQ. ID. NO. 99] specifically inhibits HPV-16 E7 mRNA levels in transformed CaSki cells.

Example S

30 Downregulation of HPV-16 E7 mRNA levels in HPV-transformed CaSki cells treated for three days with [Rp-MP/DE] [PS] [Rp-MP/DE] oligomers 3678, 3679 AND 3680

CaSki cells seeded in 60 mm dishes and cultured as described in Example R were transfected using 18 μ g/mL
35 of Lipofectamine (BRL) and 1 μ M of oligomers 3678 [SEQ.

ID. NO. 99], 3679 [SEQ. ID. NO. 100], 3680 [SEQ. ID. NO. 101] or control oligomer [SEQ. ID. NO. 102], as described before. After this first transfection the cells were washed two times with 4 mL/well of cell culture medium, and incubated for 8 hours under tissue culture conditions. The cells were transfected for 16 hours and recover under tissue culture conditions two more times. After the third transfection the cells were washed two times with 4 mL/well of PBS and total cytoplasmic RNA was extracted as described before.

The intracellular levels of HPV-16 E7 mRNA after treatment with oligomers were determined using a RNase protection assay as described in Example R. As control for specificity, the intracellular levels of GAPDH mRNA were determined by probing an aliquot of the intracellular RNA with a commercially available probe targeted to GAPDH (Ambion, pTRI-GAPDH- Human).

Phosphoimage analysis of the HPV-16 E7 protected RNA bands indicated that treatment of the cells with oligomers 3678 [SEQ. ID. NO. 99], 3679 [SEQ. ID. NO. 100] and 3680 [SEQ. ID. NO. 101] produced reduction in the amount of E7 mRNA present in the cell extracts (Figure 11). Inhibition obtained was about 70% for oligomer 3678 [SEQ. ID. NO. 99] and about 55% for oligomers 3679 [SEQ. ID. NO. 100] and 3680 [SEQ. ID. NO. 101]. No significant reduction of GAPDH levels were observed, indicating specific inhibition. Treatment of the cells with control oligomer 3268 [SEQ. ID. NO. 102] [targeted to the CAT poly(A) 3268 site] produced approximately 40% inhibition of GAPDH and approximately 25% inhibition of HPV-16 E7, indicating some non-specific effects. Overall, these results indicated that oligomers 3678 [SEQ. ID. NO. 99], 3679 [SEQ. ID. NO. 100] and 3680 [SEQ. ID. NO. 101] inhibit HPV-16 E7 mRNA levels in transformed CaSki cells. Oligomer 3678 [SEQ. ID. NO. 99] was the most potent in this model.

TABLE I

CORRELATION OF PHOSPHODIESTER OLIGO ACTIVITY WITH THE
PROPOSED SECONDARY STRUCTURE OF HPV-11 E7 mRNA

- 5 a. Prediction of the secondary structure of HPV-11 E7 around the translation initiation
codon (N469-N560)

C
5'-AGGC-ACG UUC

UUGC UGC GGAAGGUGACC

10 U U

CUGCUGGACAACAUGCAUGGAAGACUUGUUAACCCUAAAGGAUA-3'

- b. Activity of phosphodiester antisense oligos:

SEQ. ID NO.	SEQUENCE	INHIBITION
6 [2406-1]	3'-ATGTGACGACCTGTTGTA-5'	No spec. inh.
7 [2552-1]	3'-GTGACGACCTGTTGTACG-5'	No spec. inh.
8 [2458-1]	3'-ACCTGTTGTAC>A-5'	No spec. inh.
9 [2457-1]	3'-GACCTGTTGTACGTAC-5'	No spec. inh.
10 [2549-1]	3'-CGACCTGTTGTACGTACC-5'	~20% spec. inh.
11 [2553-1]	3'-CCTGTTCTACGTACCTTC-5'	~55% spec. inh.
12 [2388-1]	3'-GTTGTACGTACCTTCTG-5'	~65% spec. inh.
13 [2525-1]	3'-TGTTGTACGTACCTTCTG-5'	~70% spec. inh.
14 [2498-1]	3'-CCTGTTGTACGTACCTTCTG-5'	~70% spec. inh.

15

20

SEQ. ID NO.	SEQUENCE	INHIBITION
15 [2492-1]	3' -TACGTACCTTCTGAACAAT-5'	~80% spec. inh.
16 [2589-1]	3' -CGTACCTTCTGAACAAT-5'	~90% spec. inh.
17 [2590-1]	3' -TACGTACCTTCTGAACA-5'	~90% spec. inh.

TABLE II
ANTISENSE OLIGOMERS TARGETED TO THE TRANSLATION INITIATION
CODON OF HPV-11 E7

SEQ. ID NO.	SEQUENCE	BACKBONE
18 [2644-1]	3'-CUGUUG UACGU ACCUUCUG-5'	2' OMeRNA
19 [2624-1]	3'-CTGTTG TAGGT ACCTTCTG-5'	Racemic MP
20 [2571-1]	3'-CTGTTG TACGT ACCTTCTG-5'	75%Rp-Mp
21 [3130-2,3]	3'-CCTGTTG TACGT ACCTTCTG-5'	Rp-MP/DE
14 [2498-1]	3'-CCTGTTG TACGT ACCTTCTG-5'	(DE)
22 [2566-1]	3'-CCTGTTG TACGT ACCTTCTG-5'	(PS)
24 [2682-1]	3'-CCUGUUG (TACGT) ACCUUCUG-5'	[2' OMe] [DE] [2' OMe]
25 [2683-1]	3'-CCUGUUG (TACGT) ACCUUCUG-5'	[2' OMe] [PS] [2' OMe]
26 [2567-1]	3'-CCTGTTG (TACGT) ACCTTCTG-5'	[MP] [DE] [MP]
27 [2681-1]	3'-CCTGTTG (TACGT) ACCTTCTG-5'	[MP] [PS/DE] [MP]
28 [2687-1]	3'-CCTGTTG (TACGT) ACCTTCTG-5'	[75%RpMP] [DE] [75%RpMP]

5

10

15

SEQ. ID NO.	SEQUENCE	BACKBONE
29 [3169-1,2]	3' - CCTGTTG (TACGT) ACCTTCTG-5'	[Rp-MP/DE] [DE] ₅ [Rp-MP/DE]
30 [3214-1]	3' - CCTGTTG (TACGT) ACCTTCTG-5'	[Rp-MP/DE] [PS/DE] ₇ [Rp-MP/DE]
31 [3257-1]	3' - CCTGTTG (TACGTAC) CTTCTG-5'	[Rp-MP/DE] [PS/DE] ₇ [Rp-MP/DE]
32 [3256-1]	3' - CCTGTTG (TACGT) ACCTTCTG-5'	[Rp-MP/DE] [PS] ₅ [Rp-MP/DE]
33 [3341-1]	3' - CCUGUUG (TACGTAC) CUUGUG-5'	2' - OMe [Rp-MP/DE] [PS] ₂ -O-Me [Rp-MP/DE]
34 [3336-1]	3' - CCUGUUG (TACGTAC) CUUCUG-5'	2' - OMe [Rp-MP/DE] [PS/DE] ₂ -O-Me [Rp-MP/DE]
35 [2709-1]	3' AACAAATGGGATTTCCTATA-5'	75%Rp-MP
36 [2588]	3' - UACGUACCUUCUGAACAAU-5'	2' - OMe RNA
37 [2740]	3' AACAAUGGGGAUUUCCUAUA-5'	2' - OMe RNA
77 [3327]	3' CCTCTTG (TACGTAC) CTTCTG-5'	[MP/2' -O-MeDE] [PS] ₇ [MP/2' -O-MeDE]

TABLE III

OLIGOMERS TARGETED TO THE TRANSLATION
INITIATION CODON OF HPV-11 E1

A. Sequence comparison around the E1 translation initiation codon and splice donor site at nucleotide 847 (N820-870):

SEQ. ID. NO.	
38 [HPV-6b]	GA-C-----C-----T---
39 [HPV-11]	ACCATAACAAGGATGGCGACGATTCAAGTACAGAAATGAGGGTGGGG

B. Antisense oligomers

SEQ. ID NO.	SEQUENCE	BACKBONE
40 [2555-1]	3'-CTACCGCCTGCTAAGTCC-5'	DE
41 [2556-1]	3'-GCCTGCTAAGTCCATGTC-5'	DE
42 [2557-1]	3'-GTCCATGCTTTTACTCCCC-5'	DE
43 [3105-1]	3'-CUACCGCCUGCUAAGUC-5'	2'OMeRNA
44 [3106-1]	CAUGUCUUUUACUCCCCAG-5'	2'OMeRNA
45 [3196-1]	3'-CTACCGCCTGCTAAGTCCATG-5'	Rp-MP/DE

10

15

SEQ. ID NO.	SEQUENCE	BACKBONE
46 [3218-1]	3'-CTACCGCCTGCTAAGTCCATG-5'	[Rp-MP/DE] [PS/DE] [Rp-MP/DE]
47 [2744-1]	3'-GUCCAUG(TCTTT)UACUCCCC-5'	2'OMe-DE-2'-OMe

TABLE IV

OLIGOMERS TARGETED TO THE TRANSLATION
INITIATION CODON OF HPV-11 E2

A. Sequence comparison around the E2 translation initiation codon (N2705-2749):

SEQ. ID NO.	
48 [HPV-6b]	ATTCTGAGGACGAGGAAGATGGAAGCAATAGCCAAGCGTTTAGAT
49 [HPV-11]	-----A-----

5

B. Antisense oligomers

SEQ ID NO.	SEQUENCE	BACKBONE
50 [2167-1]	CTGCTCCTTCTACCTTCGTT	PS
51 [2550-1]	CTGCTCCTTCTACCTTCG	DE
52 [3102-1]	CCTTCTACCTTCGTTATCGG	DE
53 [3103-1]	CTACCTTCGTTATCGGTTTCG	DE
54 [2699-1]	CCUGCUCUUCUACCUUCG	2'OMeRNA
64 [3129-1]	CCUUCUACCUUCUUAUCGG	2'OMeRNA
55 [3173-1]	CUACCUUCGUUAUCGGUUCG	2'OMeRNA

10

15

SEQ ID NO.	SEQUENCE	BACKBONE
56 [3123-1]	TCCTGCTCCTTCTACCTTCG	Rp-MP/DE
57 [3124-1]	CCTTCTACCTTCGTTATCGGTTTCG	Rp-MP/DE
58 [3125-1]	CTACCTTCTTATCGGTTTCG	Rp-MP/DE
59 [3170-1]	TCCTGCT (CCTTC) TACCTTCG	[Rp-MP/DE] [DE] ₅ [Rp-MP/DE]
60 [3233-1]	CTTCTACC (TTCGT) TATCGGTTTC	[Rp-MP/DE] [DE] ₅ [Rp-MP/DE]
61 [3234-1]	CTTCTACC (TTCGT) TATCGGTTTC	[Rp-MP/DE] [PS/DE] ₅ [Rp-MP/DE]
62 [2625-1]	CCTGCTCCTTCTACCTTCG	Racemic MP
63 [2574-1]	CCTGCTCCTTCTACCTTCG	75%Rp-MP

TABLE V
OLIGOMERS TARGETED TO THE TRANSLATION
INITIATION CODON OF HPV-11 E6

A. Sequence comparison around the E6 translation initiation codon:

SEQ. ID NO.	
65 [HPV-6b (N90-142)]	-A-----GC-A-----GA-C----
66 [HPV-11 (N90-142)]	AGACGAGGCATTATGGAAAGTAAAGATGCCTCCACGCTGTGCPACATCTATAGA

B. Antisense oligomers

SEQ. ID NO.	SEQUENCE	BACKBONE
67 [2702-1]	TGCTCCGTAATACCTTCA	DE
68 [2701-1]	TAATACCTTTCATTCTA	DE
69 [2703-1]	TAATACCTTTCATTCTACGGAGG	DE
70 [2802-1]	UGCUCGUAUACCUUUA	2'OMeRNA
71 [2803-1]	UUUCUACGGAGGUGCAGA	2'OMeRNA
72 [3161-1,2,3]	CTGCTCCGTAATACCTTCA	Rp-MP/DE

SEQ. ID NO.	SEQUENCE	BACKBONE
73 [3219-1]	CUGCUC (GTAAT) ACCUUUCA	[2'OMeRNA] [PS] [2'OMeRNA]
74 [3220-1]	CUGCUC (GTAAT) ACCUUUCA	[2'OMeRNA] [DE] [2'OMeRNA]
75 [3255-1]	CTGCTCC (GTAAT) ACCTTTCA	[Rp-MP/DE] [DE] [Rp-MP/DE]
76 [3215-1]	CTGCTCC (GTAAT) ACCTTTCA	[Rp-MP/DE] [PS/DE] [Rp-MP/DE]

TABLE VI
POTENCY OF OLIGOMERS TARGETED TO HPV-11 E7

SEQ. ID NO.	BACKBONE	Tm	Cell-free assay			Vero cells Microinjection
			IC50	IC90	CAT Inhibition	
7 [2552-1]	All-DE		>1 μ M	>>1 μ M	40%, 1 μ M	N/D
11 [2553-1]	All-DE		0.03 μ M	0.2 μ M	35%, 1 μ M	N/D
12 [2388-1]	All-DE		0.03 μ M	0.2 μ M	20%, 1 μ M	N/D
15 [2492-1]	All-DE		0.005 μ M	0.05 μ M	20%, 0.1 μ M	N/D
14 [2498-1]	All-DE		~0.1 μ M	>1 μ M	45%, 1 μ M	N/D
22 [2566-1]	All-PS		~0.15 μ M	0.5 μ M	60%, 0.4 μ M	N-3+ (0.5 μ M)
26 [2567-1]	[MP] [DE] [MP]	45.6	~0.2 μ M	>>1 μ M	No inh., 1 μ M	N-2+ (0.5 μ M) C-3+ (10 μ M)
28 [2687-1]	[75%Rp-MP] [DE] [75%Rp-MP]	52.8	~0.04 μ M	1 μ M	20%, 5 μ M	N-3+ (0.5 μ M)
29 [3169-1]	[Rp-MP/DE] [DE] 5 [Rp-MP/DE]	62.6	~0.04 μ M	0.8 μ M	20%, 5 μ M	C-3+ (2 μ M)
30 [3214-1]	[Rp-MP/DE] [DE/PS] 5 [Rp-MP/DE]	61.0	~0.2 μ M	4 μ M	No inh., 10 μ M; 25%, 5 μ M	C-3+ (1 μ M)
31 [3257-1]	[Rp-MP/DE] [DE/PS] 7 [Rp-MP/DE]	60.9	~0.06 μ M	0.6 μ M	50%, 2 μ M	C-3+ (0.5 μ M)
32 [3256-1]	[Rp-MP/DE] [PS] 5 [Rp-MP/DE]	60.1	~0.25 μ M	5 μ M	No inh., 5 μ M	C-3+ (0.5 μ M)
34 [3336-1]	[Rp-MP/DE] [DE/PS] 7 [Rp-MP/DE]	66.8	~0.3 μ M	>>10 μ M	No inh., 5 μ M	N/D
33 [3341-1]	[Rp-MP/DE] [PS] 7 [Rp-MP/DE]	65.8	2 μ M	>>10 μ M	30%, 5 μ M	N/D
24 [2682-1]	[2'OMeRNA] [DE] 5 [2'OMeRNA]	72.5	~0.015 μ M	0.4 μ M	15%, 1 μ M	N-2+ (0.5 μ M) C-3+ (10 μ M)

5

10

15

SEQ. ID NO.	BACKBONE	T _m	Cell-free assay			Vero cells Microinjection
			IC ₅₀	IC ₉₀	CAT Inhibition	
25 [2683-1]	[2'OMeRNA] [PS] ₅ [2'OMeRNA]		N/D			N-3+ (0.5 μM) C-3+ (10 μM)

TABLE VII
 POTENCY OF OLIGOMERS TARGETED TO HPV-11 E7 IN A CELL BASED ASSAY

SEQ. ID. NO.	Backbone	Cell-based assay	
		IC50	IC90
29 [3169-2]	[Rp-MP/DE] - [DE] ₅ [Rp-MP/DE]	>2 μ M	>>10 μ M
30 [3214-1]	[Rp-MP/DE] - [DE/PS] ₅ [Rp-MP/DE]	0.2 μ M	1 μ M
32 [3256-1]	[Rp-MP/DE] - [PS] ₅ [Rp-MP/DE]	0.12 μ M	1 μ M
31 [3257-1]	[Rp-MP/DE] - [DE/PS] ₇ [Rp-MP/DE]	0.06 μ M	<0.3 μ M
33 [3341-1]	2' OMe [Rp-MP/DE] - [PS] ₂ 2' OMe [Rp-MP/DE]	ND	ND
34 [3336-1]	2' OMe [Rp-MP/DE] - [DE/PS] ₂ 2' OMe [Rp-MP/DE]	0.4 μ M	~2 μ M
77 [3327-1]	[MP/2' OMeDE] [PS] ₇ [MP/2' OMeDE]	0.5 μ M	~2 μ M

TABLE VIII
POTENCY OF OLIGOMERS TARGETED TO HPV-11 E7 IN MICROINJECTION IN VERO CELLS

E7 OLIGOMERS			Oligo Concentration of Microinjection Solution											
			Nuclear Injection				Cytoplasmic Injection							
			4+	3+	2+	1+	0	4+	1+	2+	1+	0		
SEQ. ID. NO	Target	Backbone												
22 [2566]	11/E7, AUG/-7	PS		0.5 μ M									0.5 μ M	
26 [2567]	11/E7, AUG/-7	MP/DE/MP			0.5 μ M				10 μ M*				0.5 μ M	
28 [2687]	11/E7, AUG/-7	75 μ RpMP/DE/75 μ RpMP		0.5 μ M									0.5 μ M	
24 [2682]	11/E7, AUG/-7	2' OMe/DE/2' OMe			0.5 μ M				10 μ M				0.5 μ M	
25 [2683]	11/E7, AUG/-7	2' OMe/PS/2' OMe		0.5 μ M					10 μ M*				0.5 μ M	
29 [3169-1]	11/E7, AUG/-7	[RpMP/DE] - [DE] - [RpMP/DE]						5 μ M	2 μ M*	0.5 μ M				
30 [3214]	11/E7, AUG/-7	[RpMP/DE] - [PS/DE]* [Rp2MP/DE]						2 μ M	1 μ M	0.5 μ M				
31 [3257]	11/E7, AUG/-7	[RpMP/DE] - [PS/DE]* [RpMP/DE]						2 μ M	0.5 μ M					
32 [3256]	11/E7, AUG/-7	[RpMP/DE] - [PS]* [RpMP/DE]						2 μ M	0.5 μ M					
18 [2644]	11/E7, AUG/-6	2' OMeRNA					1 μ M	10 μ M*						
20 [2571]	11/E7, AUG/-6	75 μ RpMP								10 μ M			0.5 μ M	
35 [2709]	11/E7, AUG/+15	75 μ RpMP									0.5 μ M each			
2571+2709														
36 [2588]	11/E7, AUG/+1	2' OMeRNA					1 μ M			10 μ M*			1 μ M	
37 [2740]	11/E7, AUG/+15	2' OMeRNA					1 μ M				10 μ M		1 μ M	
21 [3130-2]	11/E7, AUG/-7	RpMP/DE										10 μ M*		

5

10

15

20

TABLE IX
POTENCY OF OLIGOMERS TARGETED TO
HPV-11 E1 IN CELL FREE ASSAY

A.

SEQ. ID. NO.	Target	Backbone	Cell-free assay		
			IC50	IC90	CAT Inhibition
45 [3196-1]	AUG+1	Rp-MP/DE	>10 μ M	>>10 μ M	No inh. (10 μ M)

5

B.

SEQ. ID NO.	Backbone	Sequence	% inhibition of E1 (activity at 1 μ M)	% inhibition of CAT (specificity at 1 μ M)
40 [2551-1]	Phosphodiester	CTACCGCCTGCTAAGTCC	90	72
43 [3105-1]	2'OMeRNA	CUACCGCCUGCUAAGUC	50	10
41 [2556-1]	Phosphodiester	GCCTGCTAAGTCCATGTC	92	80
42 [2557-1]	Phosphodiester	GTCCATGTCCTTTTACTCCCC	98	25
47 [2744-1]	2'OMe-DE-2'OMe	GTUCCAUG (TCCTT) UACUCCCC	100	0

10

TABLE X
POTENCY OF OLIGOMERS TARGETED TO
HPV-11 E2 IN CELL FREE ASSAY

SEQ. ID NO.	Target	Backbone	Cell-free assay		
			IC50	IC90	CAT-inhibition
57 [3124-1]	AUG-5	Rp-MP/DE	5 μ M	>>10 μ M	10% (5 μ M)
59 [3170-1]	AUG-12	[Rp-MP/DE] [DE] ₅ [Rp-MP/DE]	~0.06 μ M	~1 μ M	20% (5 μ M)
60 [3233-1]	AUG-4	[Rp-MP/DE] [DE] ₅ [Rp-MP/DE]	~0.1 μ M	~1 μ M	20% (5 μ M)
61 [3234-1]	AUG-4	[Rp-MP/DE] [DE/PS] ₅ [Rp-MP/DE]	~0.1 μ M	~1 μ M	15% (10 μ M)

TABLE XI
ANTISENSE ACTIVITY OF OLIGONUCLEOTIDES TARGETED TO THE
TRANSLATION INITIATION CODON OF HPV-11 E2

SEQ. ID NO.	Target	Backbone	Sequence	TM	% inhibition of E2 (activity at 1 μ M)	% inhibition of CAT (activity at 1 μ M)
50 [2167-2]	E2, AUG-10	Phosphorothioate	CTGCTCCTTCTACCTTCGTT	59.6		
51 [2550-1]	E2, AUG-10	Phosphodiester	CTGCTCCTTCTACCTTCG		55	15
54 [2699-1]	E2, AUG-11	2'OMeRNA	UCCUGCUCCUUCUACCUUCG		70	0
62 [2625-1]	E2, AUG-11	Racemic MP	CCTGCTCCTTCTACCTTCG	42.9	0	0
63 [2574-1]	E2, AUG-11	75% Rp-MP	CCTGCTCCTTCTACCTTCG	51.8	0	0
56 [3123-1]	E2, AUG-12	Rp-MP/DE	TCCTGCTCCTTCTACCTTCG	68.4	0	0
52 [3102-1]	E2, AUG-5	Phosphodiester	CCTTCTACCTTCGTTATCGG		80	25
64 [3129-1]	E2, AUG-5	2'OMeRNA	CCUUCUACCUUCGUUAUCGG		35	0
57 [3124-1]	E2, AUG-5	Rp-MP/DE	CCTTCTACCTTCGTTATCGGTTTCG	66.4	35	0
53 [3103-1]	E2, AUG-1	Phosphodiester	CTACCTTCGTTATCGGTTTCG		100	80
55 [3173-1]	E2, AUG-1	2'OMeRNA	CUACCUUCGUUAUCGUUCG	81.1	40	0

5

10

15

TABLE XII
POTENCY OF OLIGOMERS TARGETED TO HPV-11 E2 IN MICROINJECTION TO VERO CELLS

Microinjection Summary												
E2 OLIGOMERS				Oligo Concentration of Microinjection Solution								
SEQ. ID. NO	Target	Backbone	Nuclear Injection				Cytoplasmic Injection					
			4+	3+	2+	1+	0	4+	3+	2+	1+	0
60 [3233]	11/E2.AUG/-4	[RpMP/DE] - [DS], [RpMP/DE]						2 μ M	1 μ M	0.5 μ M		
61 [3234]	11/E2.AUG/-4	[RpMP/DE] - [PS/DE], [RpMP/DE]						1 μ M	0.5 μ M			
59 [3170]	11/E2.AUG/-12	[RpMP/DE] - [DB], [RpMP/DE]						2 μ M	1 μ M	0.5 μ M		
63 [2574-1]	11/E2.AUG/-10	758RpMP						2 μ M*	1 μ M	0.5 μ M		
54 [2699-1]	11/E2.AUG/-10	2'MeRNA						2 μ M				
56 [3123-1]	11/E2.AUG/-12	RpMP/DE									10 μ M	2 μ M
57 [3124-1]	11/E2.AUG/-5	RpMP/DE							2 μ M 10 μ M*			
58 [3125-2]	11/E2.AUG/-1	RpMP/DE						10 μ M	2 μ M 5 μ M	1 μ M	0.5 μ M	

TABLE XIII
POTENCY OF OLIGOMERS TARGETED TO HPV-11 E6 IN CELL FREE ASSAY OF OLIGONUCLEOTIDES

TABLE A

SEQ. ID. NO.	Backbone	Sequence	% inhibition of E6 (activity at 1 μ M)	% inhibition of CAT (specificity at 1 μ M)	TM
68 [2701-1]	Phosphodiester	TAATACCTTTCATTCTA	5	0	
69 [2703-1]	Phosphodiester	TAATACCTTTCATTCTACGAGG	35	15	
67 [2702-1]	Phosphodiester	TGCTCCGTAATACCTTTCA	30	5	
70 [2802-1]	2'OMeRNA	UGCUCGUAUACCUUCA	65	0	75.7
72 [3161-1]	Rp-MP/DE	CTGCTCCGTAATACCTTTCA	55	0	60.6

5

10 TABLE B

Cell-free assay					
SEQ. ID. NO.	Target	Backbone	IC50	IC90	CAT inhibition
72 [3161-1]	AUG-10	Rp-MP/DE	1 μ M	>>10 μ M	10% (10 μ M)
75 [3255-1]	AUG-10	[Rp-MP/DE] [DE] ¹ [Rp-MP/DE]	1 μ M	5 μ M	No inh. (10 μ M)
76 [3215-1]	AUG-10	[Rp-MP/DE] [DE/PS] ² [Rp-MP/DE]	0.3 μ M	2 μ M	no inh. (10 μ M)

TABLE XIV

SEQ. ID. NO.	Backbone	Oligo concentration (μ M)			
		0.01	0.1	1	10
26 [2567-1]	[MP] - [DE] ₅ - [MP]	2	15	50	80
29 [3169-1]	[Rp-MP/DE] - [DE] ₅ [Rp-MP/DE]	7	45	85	100
30 [3214-1]	[Rp-MP/DE] - [PS/DE] ₅ - [Rp-MP/DE]	2	20	50	80
31 [3257-1]	[Rp-MP/DE] - [PS/DE] ₇ - [Rp-MP/DE]	4	40	75	100
33 [3341-1]	2'OMe [Rp-MP/DE] - [PS] ₇ - 2'OMe [Rp-MP/DE]	5	40	60	60
34 [3336-1]	2'OMe [Rp-MP/DE] - [PS/DE] ₇ - 2'OMe [Rp-MP/DE]	5	50	60	65

Results are percentage of cleavage of E7 mRNA. Estimated values were obtained by visual inspection of the gel.

TABLE XV
ANTISENSE OLIGOMERS TARGETED TO THE TRANSLATION
INITIATION CODON OF HPV-16 E7

SEQ. ID NO.	SEQUENCE	BACKBONE
99 [3678-1]	5' - GCATGA (TTACA) GCTGGGTTT - 3'	[Rp-MP/DE] [PS], [Rp-MP/DE]
100 [3679-1]	5' - CCATGCAT (GATT) CAGCTGG - 3'	[Rp-MP/DE] [PS], [Rp-MP/DE]
101 [3680-1]	5' - GTATCTCC (ATGCA) TGATTACAG - 3'	[Rp-MP/DE] [PS], [Rp-MP/DE]
Control 102 [3268]	5' - CCTCATTTT (ATTAG) GAAAGGACAG - 3'	[Rp-MP/DE] [PS], [Rp-MP/DE]

Claims

1. An oligomer having either at least one internucleosidyl linkage which is not a phosphodiester linkage or at least one nucleosidyl unit which has a 2'-substituted ribosyl moiety and which has from about 14 to about 35 nucleosidyl units and which has a nucleoside base sequence complementary to a target region of an mRNA or pre-mRNA (a) which target region includes a sequence selected from a translation initiation codon, a splice donor site, a splice acceptor site, a coding region, a polyadenylation signal, a 3'-untranslated region and a 5'-untranslated region and (b) wherein the mRNA or pre-mRNA codes for a human papilloma virus gene selected from E1, E2, E6 and E7.
2. An oligomer according to claim 1 which is capable of decreasing expression of the virus gene by at least about 75 percent.
3. An oligomer according to claim 2 wherein said gene is E6 or E7.
4. An oligomer according to claim 2 wherein said target region is about -20 to +20 nucleosides of the splice donor site, about -20 to about +20 nucleosides of the splice acceptor site or about -25 to about +35 nucleotides of the initiation codon.
5. An oligomer according to claim 4 wherein said HPV is HPV-6b or HPV-11.
6. An oligomer according to claim 5 wherein said gene is E6 or E7.
7. An oligomer according to claim 4 wherein said gene is E6 or E7.

8. An oligomer according to claim 2 comprising an RNase H-activating region and a non-RNase H activating region, wherein

the RNase H-activating region comprises a segment of
5 at least three consecutive 2'-unsubstituted nucleosides linked by charged internucleoside linkage structures,

the non-RNase H-activating region comprises a segment of at least two linked nucleosides, at least one of the linkages in said non-RNase H-activating region
10 being chirally-selected.

9. An oligomer according to claim 8 wherein said RNase H-activating region comprises between five and about nine consecutive linked nucleosides.

10. An oligomer according to claim 9 wherein the
15 charged linkage structures in said RNase H-activating region are selected from the group consisting of phosphodiester linkages, phosphorodithioate linkages and phosphorothioate linkages.

11. An oligomer according to claim 10 wherein the
20 segment of charged linkage structures in said RNase H-activating region comprises a mixed charged linkage sequence including at least two different charged linkage structures.

12. An oligomer according to claim 11 wherein the
25 oligomer is complementary to at least a portion of 5'-GGACAACAUCAUGGAAGACUUGUUACCC-3' [SEQ. ID. NO. 95].

13. An oligomer according to claim 2 wherein the oligomer is complementary to at least a portion of 5'-GGACAACAUCAUGGAAGACUUGUUACCC-3' [SEQ. ID. NO. 95].

14. An oligomer according to claim 13 which has from about 18 to about 24 nucleosidyl units.

15. An oligomer according to claim 11 which has the nucleoside sequence 3'-CCTGTTGTACGTACCTTCTG-5' [SEQ. ID. NO. 96].

16. An oligomer according to claim 1 comprising an RNase H-activating region and a non-RNase H activating region, wherein

the RNase H-activating region comprises a segment of at least three consecutive 2'-unsubstituted nucleosides linked by charged internucleoside linkage structures,

the non-RNase H-activating region comprises a segment of at least two linked nucleosides, at least one of the linkages in said non-RNase H-activating region being chirally-selected.

17. An oligomer according to claim 16 wherein said RNase H-activating region comprises between five and about nine consecutive linked nucleosides.

18. An oligomer according to claim 17 wherein the charged linkage structures in said RNase H-activating region are selected from the group consisting of phosphodiester linkages, phosphorodithioate linkages and phosphorothioate linkages.

19. An oligomer according to claim 18 wherein the segment of charged linkage structures in said RNase H-activating region comprises a mixed charged linkage sequence including at least two different charged linkage structures.

20. An Oligomer according to claim 1 having phosphonate internucleosidyl linkages selected from the

group consisting of lower alkylphosphonate internucleosidyl linkages of 1 to 3 carbon atoms and lower alkylphosphonothioate internucleosidyl linkages of 1 to 3 carbon atoms which are mixed with non-phosphonate internucleosidyl linkages wherein the phosphonate linkages are interspersed between single non-phosphonate internucleosidyl linkages in a ratio of from about 1 to 1 to about 1 to 4 non-phosphonate linkages to phosphonate linkages and wherein the Oligomer is substantially complementary to the RNA target sequence.

21. An oligomer according to claim 20 wherein said phosphonate linkages are chirally pure Rp methylphosphonate linkages.

22. An oligomer according to claim 21 wherein said non-phosphonate linkages are selected from the group consisting of phosphodiester, phosphotriester, phosphorothioate, phosphorodithioate, phosphoramidate, phosphorofluoridate, boranophosphate, formacetal and silyl.

23. An oligomer according to claim 22 wherein the nucleosides of said oligomer have 2'-O-methyl ribosyl groups as sugar moieties.

24. An oligomer complementary to an mRNA or pre-mRNA encoding E7 of an HPV which comprises about 14 to about 35 nucleosidyl units and which has a nucleoside base sequence which is complementary to a portion of the mRNA or pre-mRNA in the region of -25 to +35 wherein +1 to +3 is the initiation codon.

25. An oligomer which has a nucleoside base sequence complementary to an mRNA or pre-mRNA coding for E6 or E7 of an HPV having a pseudoknot region wherein

the oligomer has from about 14 to about 35 nucleosidyl units and is complementary to a target sequence of the mRNA or pre-mRNA immediately 5'- or immediately 3'- to the pseudoknot region.

- 5 26. An oligomer according to claim 25 wherein said target sequence is immediately 3'- to the pseudoknot region.

27. An oligomer according to claim 26 wherein the pseudoknot region has a sequence selected from
10 5'-AGGCAGCUUCCCAGUGGAAGGGUCGUUGCUUCUGCU-3'
or
5'-AGGCGCGGUUCGUGGAAGGGUCGCGCCUCUGCU-3' [SEQ. ID NOS. 97 and 98].

28. An oligomer which has from about 14 to about 35
15 nucleosidyl units and which has a nucleoside base sequence complementary to a target region of an mRNA or pre-mRNA (a) which target region includes a sequence selected from a translation initiation codon, a splice donor site, a splice acceptor site, a coding region, a
20 polyadenylation signal, a 3'-end of an untranslated region and a 5'-end of an untranslated region and (b) wherein the mRNA or pre-mRNA codes for a human papilloma virus gene selected from E1, E2, E6 and E7.

29. An oligomer according to claim 28 wherein said
25 target region is about -20 to +20 nucleosides of the splice donor site, about -20 to about +20 nucleosides of the splice acceptor site or about -25 to about +35 nucleotides of the initiation codon.

30. An oligomer according to claim 29 wherein said
30 HPV is HPV-6b or HPV-11.

31. An oligomer according to claim 30 wherein said gene is E6 or E7.

32. An oligomer according to claim 29 wherein said gene is E6 or E7.

5 33. An oligomer according to claim 28 comprising an RNase H-activating region and a non-RNase H activating region, wherein

the RNase H-activating region comprises a segment of at least three consecutive 2'-unsubstituted nucleosides
10 linked by charged internucleoside linkage structures,
the non-RNase H-activating region comprises a segment of at least two linked nucleosides, at least one of the linkages in said non-RNase H-activating region being chirally-selected.

15 34. An oligomer according to claim 33 wherein said RNase H-activating region comprises between five and about nine consecutive linked nucleosides.

35. An oligomer according to claim 34 wherein the charged linkage structures in said RNase H-activating
20 region are selected from the group consisting of phosphodiester linkages, phosphorodithioate linkages and phosphorothioate linkages.

36. An oligomer according to claim 35 wherein the segment of charged linkage structures in said RNase H-
25 activating region comprises a mixed charged linkage sequence including at least two different charged linkage structures.

37. An oligomer according to claim 36 wherein the oligomer is complementary to at least a portion of
30 5'-GGACAACAUGCAUGGAAGACUUGUUACCC-3' [SEQ. ID. NO. 95].

38. An oligomer according to claim 28 wherein the oligomer is complementary to at least a portion of 5'-GGACAACAUGCAUGGAAGACUUGUUACCC-3' [SEQ. ID. NO. 95].

39. A oligomer according to claim 28 having
5 phosphonate internucleosidyl linkages selected from the group consisting of lower alkylphosphonate internucleosidyl linkages of 1 to 3 carbon atoms and lower alkylphosphonothioate internucleosidyl linkages of 1 to 3 carbon atoms which are mixed with non-phosphonate
10 internucleosidyl linkages wherein the phosphonate linkages are interspersed between single non-phosphonate internucleosidyl linkages in a ratio of from about 1 to 1 to about 1 to 4 non-phosphonate linkages to phosphonate linkages and wherein the Oligomer is
15 substantially complementary to the RNA target sequence.

40. An oligomer according to claim 39 wherein said phosphonate linkages are chirally pure Rp methylphosphonate linkages.

41. An oligomer according to claim 40 wherein said
20 non-phosphonate linkages are selected from the group consisting of phosphodiester, phosphotriester, phosphorothioate, phosphorodithioate, phosphoramidate, phosphorofluoridate, boranophosphate, formacetal and silyl.

25 42. An oligomer according to claim 41 wherein the nucleosides of said oligomer have 2'-O-methyl ribosyl groups as sugar moieties.

43. An oligomer according to claim 42 wherein the oligomer is complementary to at least a portion of
30 5'-GGACAACAUGCAUGGAAGACUUGUUACCC-3' [SEQ. ID. NO. 95].

44. An oligomer according to claim 39 wherein the oligomer is complementary to at least a portion of 5'-GGACAACAUGCAUGGAAGACUUGUUACCC-3' [SEQ. ID. NO. 95].

45. An oligomer according to claim 28 which is
5 capable of decreasing expression of the virus gene by at least about 75 percent.

46. An oligomer according to claim 4 wherein said HPV is HPV-16.

47. An oligomer according to claim 46 wherein said
10 gene is E6 or E7.

48. An oligomer according to claim 29 wherein said HPV is HPV-16.

49. An oligomer according to claim 48 wherein said gene is E6 or E7.

50. An oligomer according to claim 34 wherein said
15 HPV is HPV-16.

51. An oligomer according to claim 50 wherein said gene is E6 or E7.

52. An oligomer according to claim 39 wherein said
20 HPV is HPV-16.

53. An oligomer according to claim 52 wherein said gene is E6 or E7.

Figure 1
Polycistronic E6/E7 mRNA and monocistronic E7 mRNA found in
HPV-6b or HPV-11 condylomas

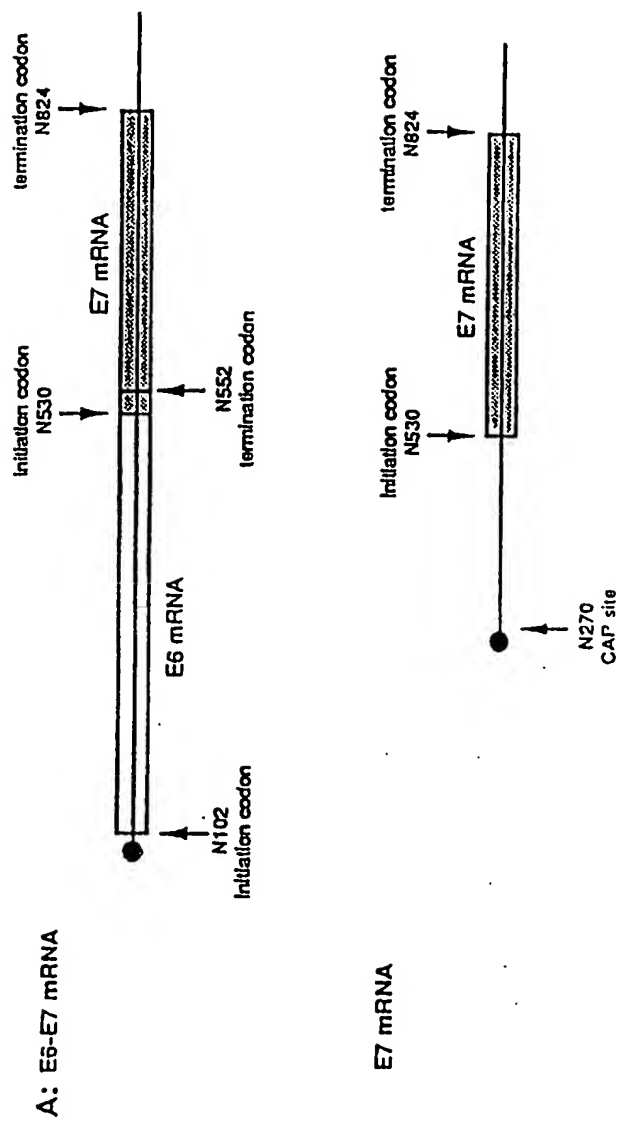


Figure 2 A

Alignment of HPV sequences around the E6/E7 boundary

```

<0--<1-----<2-----<1>-----<2>
470 480 490 500 510 520 530
HPV 11: GGAAGGCACGCTTCATTAATAA--CCAGTGAAGGTCGTTGCTTACA-----CTGCTGGAC-----AACATGCAAT
HPV 6 : ACCAAGCGCGGTTTCATTAAGCTAAATTG-TACGTGAAGGTCGCTGCTTACA-----CTGCTGGAC-----AACATGCAAT
HPV 44: GACAAGCGCGGTTTCATTAATTAACAAGA-TACCTGAAGGTCGCTGCTTCCA-----TTGTTGGAC-----ATCATGCAAT
HPV 43: CAAAAGGCACAATTCTTTAAATACATAGCGTGTGGAAGGATACCTTACA-----TTGCTGGAA-----ATCATGCAAT
cons#1 ...AAGGCRCg.TTC.T.Aa.TA.A..R-....TGGAAggtcgtctctTaCA---YTGCTGGAC-----A.CATGCAAT

<1-----<2-----<1>-----<2>
470 480 490 500 510 520 530
HPV 35: GAAAAAAGCGATTCCTAATACATCGGTGG-ACGGTGGACAGGTCGGTGTATGTC-----CTGTTGGAA-{27}ATCATGCAAT
HPV 16: AAAAAAGCAAGATTCCATAATATAAGGGG-TCGGTGGACCGGTCGATGTATGTC-----TTGTTGCAG-{33}ATCATGCAAT
HPV 31: AAAAAAGAAACGATTCCACATAGGAGG-AAGGTGGACAGGACGTTGCTATAGC-----ATGTTGGAG-{27}AACATGCGT
HPV 33: TTAACAACGATTTCAATATTTTCGGG-TCGTGGGCGGCGCTGTCGGC-----GTGTTGGAG-{39}GCCATGAGA
HPV 18: GAAAAACGACGATTTCAACATAGTCGG-GCATATAGAGGCCAGTGCCTATTC-----GTGCTGCAA-{54}AGTATGCAAT
HPV 39: AGCAACGAAGATTTCATATAAATAGCAGG-AAGCTATACAGGACAGTGTGACG-----GTGCTGCAAT-{52}GATATGCGT
HPV 51: GAAAAAAGGTTTCATGAATAGCGGG-ACGTTGGACGGGGCAATGCGCTAA-----TTGCTGGCA-{38}GCCATGCGT
HPV 58: TTAACAACGATTTCAATATTTTCGGG-TCGTTGGACAGGGCGCTGTGCACTGTGTTGGAGACCC{39}CCCATGAGA
HPV 2a: GGAACAAGCGTTTCCACAACATATCAGG-CCGTTGGACGGGACATTCATGAA-----CTGCGGGTC-----ATCATGCAC
cons#2 ..aAA..Ra.GrTTYCataA.AT.....GG-..R.Tg.acRGG.CR.Tgy.....Tgy.Gr.....YATG.Ry

```

Figure 2 B
Pseudoknot structure present in HPV E6/E7 mRNA

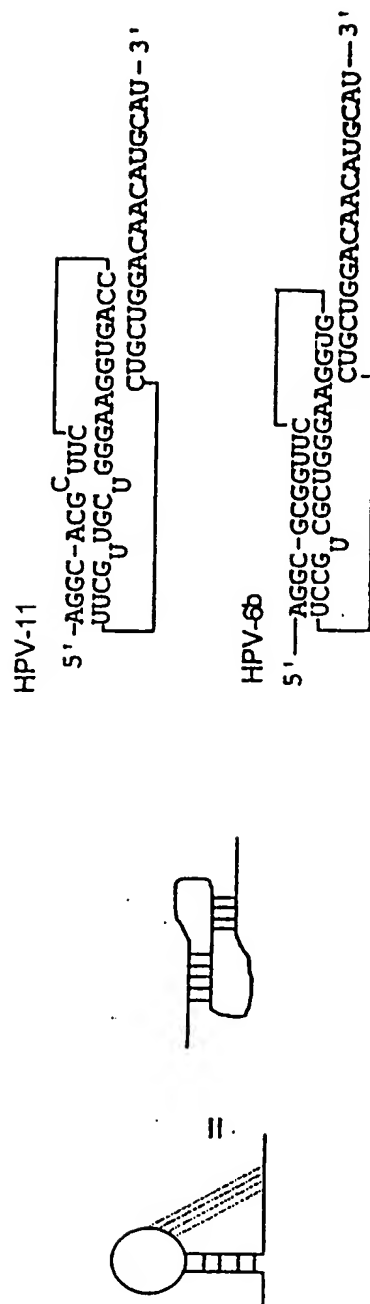


Figure 3

Chimeric oligomers inhibit translation of E7 mono and polycistronic mRNA

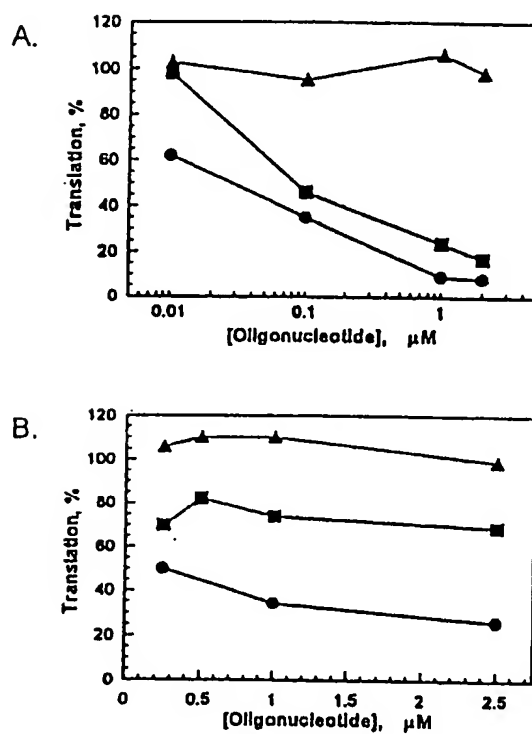


Figure 4

Inhibition of expression of E6 and E7 proteins by a single oligonucleotide

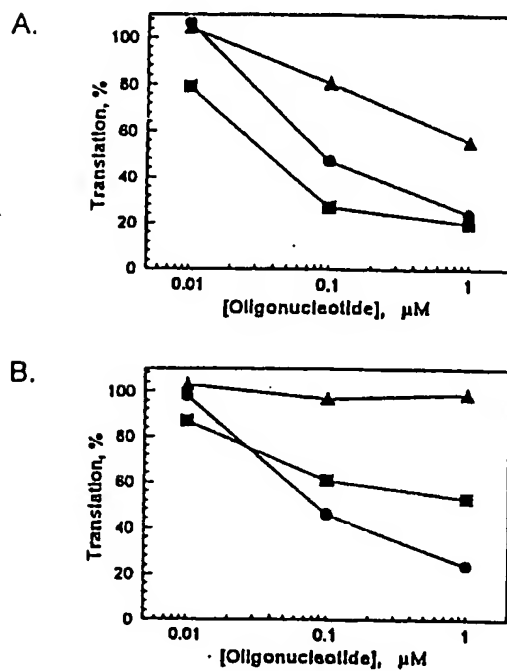


Figure 5

Inhibition of HPV-11 E7 protein expression in transiently transfected COS-7 cells

Dose response with oligomer # 3256, [Rp-MP/DE][PS]₅[Rp-MP/DE]

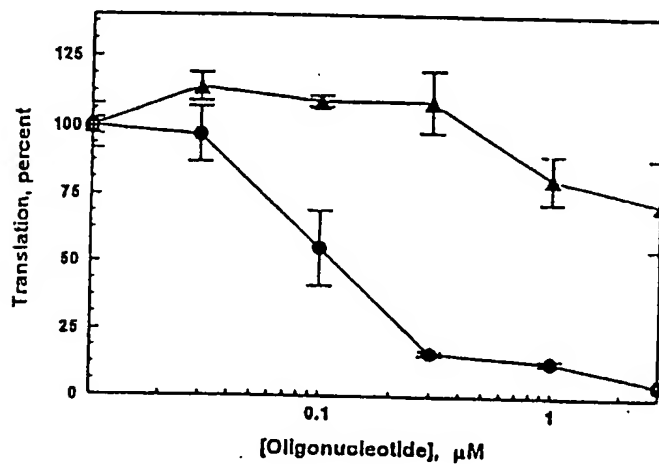


Figure 6

Downregulation of E7 mRNA levels in COS-7 cells treated with [Rp-MP/DE][PS][Rp-MP/DE]

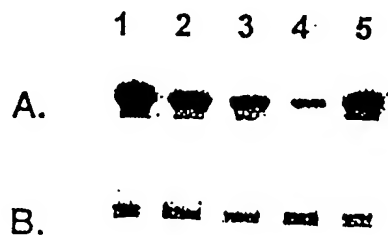
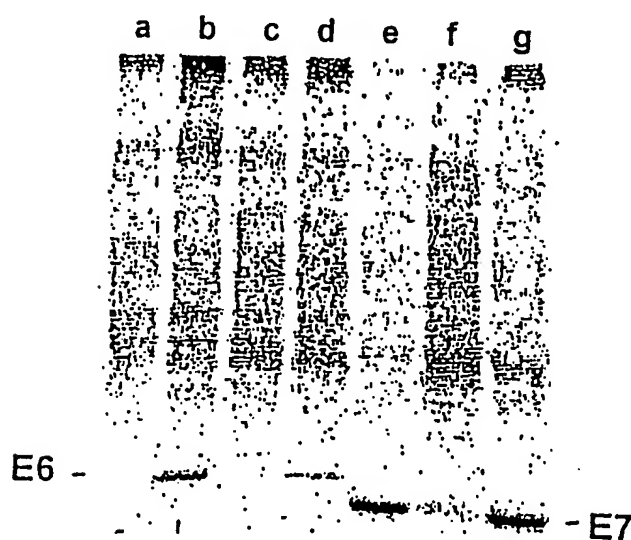


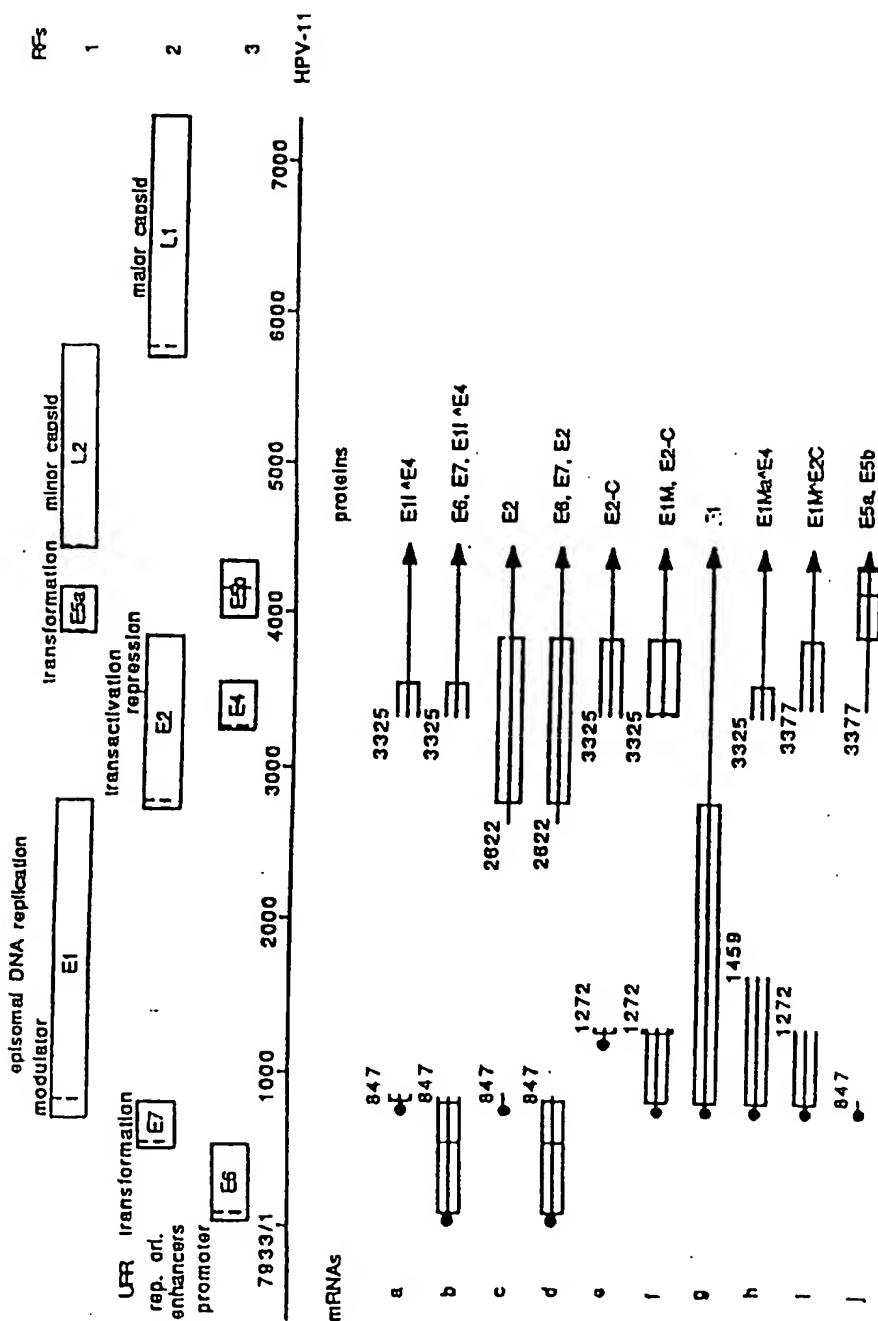
Figure 7

*Inhibition of expression of E6 and E7 proteins
by a single chimeric oligonucleotide*



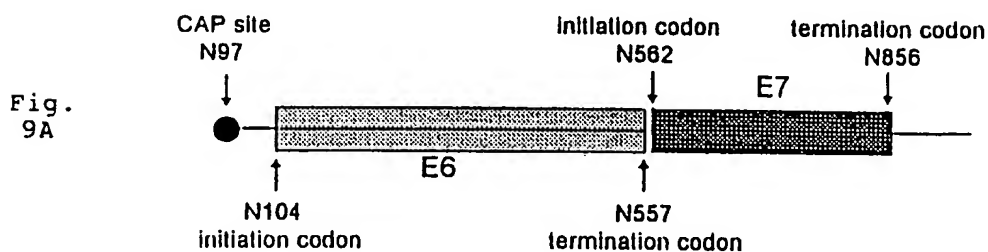
Best Available Copy

FIGURE 8

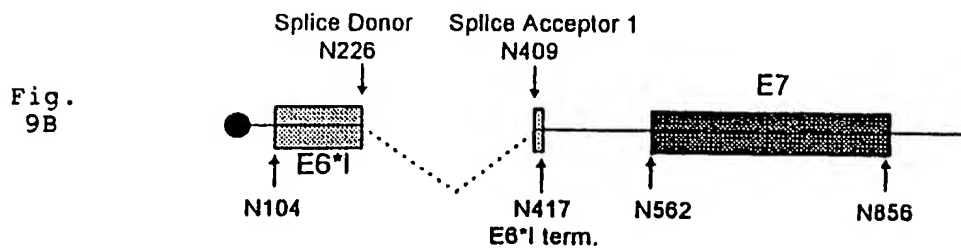


*Structure of E6/E7 transcripts found in
HPV-16 transformed CaSki cells*

E6/E7 mRNA



E6*I/E7 mRNA



E6*II/E7 mRNA

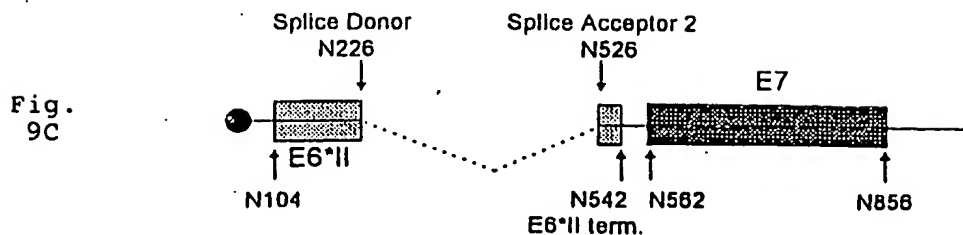
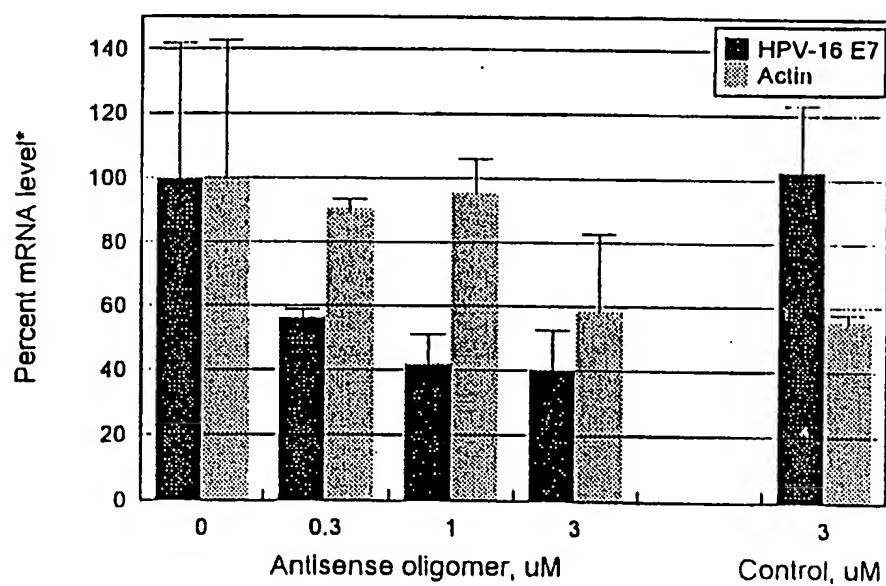


Figure 10

*Downregulation of HPV-16 E6/E7 mRNA levels in
HPV-transformed CaSki cells*

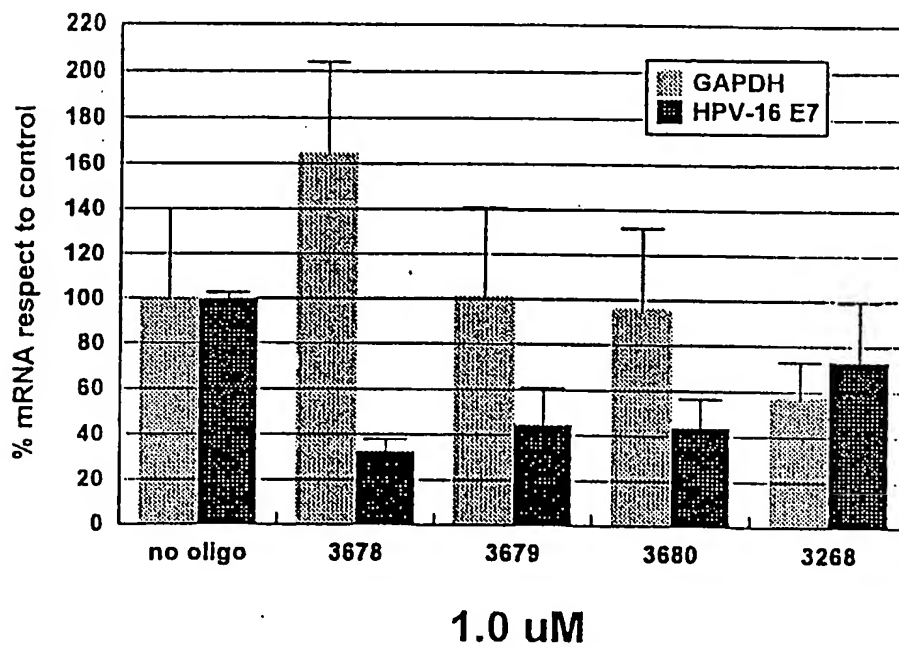
Dose-response with oligomer 3678, [RpMP/DE][PS]5[RpMP/DE]



* Compared to cells treated only with Lipofectamine

Figure 11

Downregulation of HPV-16 E6/E7 mRNA levels in HPV-transformed CaSki cells treated with [Rp-MP/DE][PS][Rp-MP/DE] oligomers



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/05179

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 31/70; C07H 21/00; C12N 15/11

'S CL : 435/6, 91.1; 536/23.1, 24.5; 514/44

A. According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1; 536 23.1, 24.5; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG; BIOSIS, MEDLINE, CAS, Derwent Biotechnology Abstracts

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A, 93/20095 (CROOKE ET AL.) 14 October 1993, see entire document.	1-12, 16-26, 28-36, 39-42, 45-53
Y	WO, A, 91/08313 (CROOKE ET AL.) 13 June 1991; see entire document.	1-12, 16-26, 28-36, 39-42, 45-53
Y	Int. J. Cancer, Vol. 51, No. 5, issued 09 July 1992, Von Knebel Boeberitz et al., "Inhibition of tumorigenicity of cervical cancer cells in nude mice by HPV E6-E7 anti-sense RNA", pages 831-834, see entire document.	1-12, 16-26, 28-36, 39-42, 45-53



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

11 JULY 1995

Date of mailing of the international search report

09 AUG 1995

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

John L. LeGUYADER

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/05179

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Clinical Research, Vol. 39, No. 4, issued 1991, Cowsert et al., "Antisense oligonucleotides as inhibitors of papillomavirus", page 818A, see entire abstract.	1-12, 16-26, 28-36, 39-42, 45-53
Y	Nucleic Acids Research, Vol. 19, No. 15, issued 1991, Storey et al., "Anti-sense phosphorothioate oligonucleotides have both specific and non-specific effects on cells containing human papillomavirus type 16", pages 4109-4114, see entire document.	1-12, 16-26, 28-36, 39-42, 45-53
Y	Antimicrobial Agents and Chemotherapy, Vol. 37, No. 2, issued Feb. 1993, Cowsert et al., "In vitro evaluation of phosphorothioate oligonucleotides targeted to the E2 mRNA of papillomavirus: potential treatment for genital warts", pages 171-177, see entire document.	1-12, 16-26, 28-36, 39-42, 45-53